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BIOREACTOR FOR COLD HYDROLYSIS & FERMENTATION OF WHEAT STARCH

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Bioreactor for Cold Hydrolysis of Wheat Starch

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EXCUTIVE SUMMARY

Wheat is an abundant agriculture product of Saskatchewan. Fuel grade ethanol production using wheat as a feedstock diversifies traditional food-based markets and so increases wheat demand which helps to stabilize world prices. When ethanol is blended with gasoline (gasohol), it lowers the carbon oxide emissions from automobiles, benefiting our environment.

A key step in ethanol fermentation is the hydrolysis of wheat starch, which commercially involves a mash cooking operation followed by high temperature enzymatic liquefaction. This final report summaries our efforts over the last two years in developing a new, cold starch hydrolysis technology to replace the traditional energy intensive procedures. The evolution of our study eventually led to a single stage conversion of starch to ethanol process, making valuable food-quality proteins as a byproduct.

To carry out the experimental research, we have designed and fabricated a membrane bioreactor. It consists of a 5 L, jacketed glass vessel equipped with temperature, pH and level controllers; a microfiltration hollow fiber membrane filter to separate and recycle unused starch and microbial cells; and an ultrafiltration hollow fiber filter to separate and recycle unused enzymes.

We began our investigation by demonstrating that cold starch hydrolysis in the scaled-up membrane bioreactor followed the same kinetics reported earlier by us in a 25 mL reactor. With 30 g/L barley malt amylase, 70 % of a 100 g/L starch feedstock was

hydrolyzed at 45 °C in 10 h; after 30 h, the starch conversion increased to 90 %, producing a hydrolysate with 75 g/L of glucose.

Due to enzyme inactivation, continuous starch hydrolysis in the bioreactor slowly decayed, creating a build-up of undissolved starch particles with time. By supplementing fresh enzyme, a continuous hydrolysis of 100 g/L starch feed was maintained over 270 h. In the first 150 h, the average enzyme productivity was about 0.04 gram starch per gram enzyme per hour (gS/gE/h) but due to enzyme decay it decreased after that time period.

We then studied enzyme inactivation kinetics and the effect of product inhibition. The loss of activity was 1.1 % per hour at 45 °C and 3.2 % per hour at 55 °C. The inhibition model predicted that when the cumulative monosaccharide and disaccharide concentration reached 100 g/L (as desired for fermentation), the enzyme activity would drop 50 % compared to no product inhibition. The implication of this discovery was that the operation of a continuous, well-mixed reactor would not be a favorable design strategy. Batch or fed batch modes of operation should be utilized for cold starch hydrolysis.

We then developed a sequential fed batch process for cold starch hydrolysis. Wheat starch hydrolysis was first carried out in a batch reactor. After reaching some desired conversion, the reaction was stopped and the hydrolysate was filtered through our membrane apparatus. This meant that unused starch and all of the enzyme was retained in the reactor and reused in subsequent batches. When charging 45 g of barley amylase to the bioreactor and reusing it for the second and third batches, 810 g of starch was hydrolyzed at 40 °C in 114 h. The average glucose concentration in the three batches of hydrolysates ranged from 52 to 62 g/L, whereas the total sugar was between 83 to 110

g/L. The enzyme hourly productivity reached 0.16 gS/gE/h, a four fold improvement compared with the best productivity attained during our continuous hydrolysis testing.

The starch hydrolysates were then successfully used in ethanol fermentation studies. We have shown that in a batch fermentor, a 105 g/L sugar solution was fermented to 51 g/L ethanol within 22 h, at a 94 % theoretical yield or a glucose to ethanol yield of 0.48 g/g. When conducting continuous fermentation with a 90 g/L glucose feed and at a dilution rate of 0.1 h^{-1}, we have obtained a 93 % average glucose conversion with an ethanol yield of 0.49 g/g.

We have also tested whole wheat flour (with bran) and bran-free flour as feedstocks for cold starch hydrolysis. In general, coarse wheat bran compromised both the hydrolysis and membrane filtration processes. It cannot be successfully used for cold starch hydrolysis. Debranning of wheat increases the starch concentration in the feedstock which in turn will increase ethanol production and reduce energy consumption.

Growth of *Candida famata* yeast contaminated both whole wheat and bran-free flour hydrolyses. *C.f.* converted fermentable sugars to acids, thus severely lowering the overall ethanol yields. Although using autoclaved flour solved this problem for a short period, it did not completely eliminate the problem. It was difficult even in the laboratory to destroy *C.f.* cells and so contamination would be a major hurdle for cold hydrolysis in commercial, continuous operations.

We then combined hydrolysis and fermentation in a single vessel by charging the hydrolyzing enzymes and brewing yeast simultaneously. The one step process alleviated product inhibition of enzymes and reduced operation cost. Using 130 g/L of wheat starch or flour as substrates at 30 to 35 °C, about 95 % of the starch was directly converted in 24

h to produce ethanol concentration of 61 (from starch) and 51 (from flour) g/L, respectively. The glucose to ethanol yields were again 95 % of the theoretical yield. More significantly, because brewing yeast outgrew *C. fomata*, contamination was suppressed during the simultaneous hydrolysis and fermentation (SHF) even with flour feedstock.

The simultaneous hydrolysis fermentation was next studied in sequential fed batch mode. Both enzymes and yeast were recycled in the membrane bioreactor. The concentrated yeast cells further accelerated the fermentation rate. We have shown an enzyme loading of 15 g/L barley malt amylase can be reused for at least three sequential, 24 h period, batches. For each batch with a loading of 130 g/L starch, the average daily ethanol production per fermentation volume was 63 g/L/day. The process efficiency was around 86 % from starch to ethanol. Conversion of glucose as it was formed also reduced the broth viscosity making it easier to filter than starch hydrolysates. The microfiltration flux increased to 37 L (broth)/m^2/h.

We finally performed an economic evaluation, based on our sequential batch, cold simultaneous hydrolysis fermentation process. The process was designed to produce 10 million liter per year of ethanol similar to the Pound Maker plant now at Lanigan. Using debranned wheat flour and conducting both hydrolysis and fermentation below 35 °C, the retentate from the membrane bioreactor is not denatured and can be marketed as food protein similar to wheat gluten. The entire plant requires $8.33 million total capital investment with an annual operating cost of $5.24 million. From the sales of ethanol, food protein, and bran as animal feed, the plant annual revenue is estimated at $10.9 million. This results in a Discounted Cash Flow Rate of Return of 46 % and a Payback

Period of 27 months, indicating that this process is worthy of further investigation and some aspects should be advanced to Pilot Scale development.

1. INTRODUCTION

Saskatchewan is the major producer of wheat, barley and canola seeds in Canada. Each year, approximately 12 million tonnes of Canadian wheat is grown in the Province. About 10 % of Saskatchewan agricultural commodities are presently processed in the Province, the other 90 % are directly sold or exported as raw materials. In comparison, the percentage of processed agricultural products amounts to 60 % in the Provinces of Ontario and Quebec. To stimulate further economical growth, both the Saskatchewan government and private business sectors have realized and emphasized the importance of diversifying our traditional agriculture practices. More emphasis needs to be placed on the manufacture of value-added products which utilize our own agricultural feedstocks. One such diversifying operation is the fermentation of ethanol from grain starch.

Fermentation of grain, corn, barley and wheat was the principle method for production of industrial ethanol before the era of the petrochemical industry. In the late 1980s, ethanol production from grain came to be viewed as a way to increase demand for corn and wheat, stabilizing the commodity price in both the USA and Canada. In the 1990s, the environmental benefits of oxygenated fuels such as ethanol-gasoline blends (gasohol) are also recognized as a way to lower carbon oxide emissions from automobiles.

Fermentation ethanol can be produced from grain by two standard methods: dry and wet milling (Lewis, 1996). In the dry milling process, ground whole grain is directly used for hydrolysis to produce fermentable sugars, which are next fermented to ethanol. In the wet milling process, the grain is washed after grinding to separate germ, fiber and

gluten from the starch. Only the starch slurry is used for hydrolysis. Recently, it has also been proposed that a preprocessed flour, with fibers already removed from the grain, should be used as the preferred feedstock (Sosulski et al. 1997).

The majority of industrial starch hydrolysis processes involve continuous jet mash cooking and liquefaction by microbial bacterial α-amylase enzymes at temperatures over 90 °C. This is followed by saccharification by glucoamylase either in a separate step or simultaneously during fermentation. Since the discovery of heat stable *Bacillus licheniformis* α-amylase, this high temperature (up to 140 °C) jet cooking process has become a very efficient, mature technology. However, in 1987, Han et al. achieved a 17% energy saving in the overall production of ethanol by lowering the cooking temperature. Mikuni et al. (1987) also reported that energy saving could reach as much as 40 % by completely eliminating the cooking step. The removal of the cooking step also has the potential to increase the value of the co-products since valuable grain protein would be spared from thermal denaturing. In addition, there are other benefits such as extended use of enzyme and the possibility of a single stage, starch to ethanol process.

In large scale operations, hydrolyzing enzymes, mainly α-amylase and glucoamylase, are co-fed to the reactor with the substrates. These enzymes lose their activities after completing one residence time cycle. There are two methods to extend the use of enzymes. One involves immobilization on a carrier or membrane. The other option is ultrafiltration of the medium to retain the soluble enzymes. Packed columns of immobilized glucoamylase have been tested industrially for starch processing and it is reported that the enzyme has lasted for 6 months (Daniels, 1986). α-Amylase immobilization for liquefaction (Ju et al. 1995) is also reported. Immobilization

introduces mass transfer limitations, and enzymes always lose activity during the immobilization procedure. In comparison, membrane filtration of soluble enzyme does not have these difficulties and it is easy to implement with existing technology.

Previously, we have demonstrated in a small batch reactor that at pH 4.5 and 44 °C, barley malt α-amylase could efficiently hydrolyze wheat starch granules into glucose (ADF Project No. 94000287, 1995; also Textor et al., 1997). Barley amylase was found to be superior than bacterial (*Bacillus* species) and fungal (*Aspergillus oryzae*) α-amylases.

The present project evolved out of the success of our previous ADF project. The objective is to develop a feasible, commercial hydrolysis process at temperatures below the starch gelatinization temperature to manufacture fermentation ethanol from Saskatchewan wheat. To accomplish this goal, we have divided our work into the following areas:

1) Design a membrane ultrafiltration bioreactor that can retain soluble enzyme for reuse.
2) Develop the process of cold hydrolysis using wheat starch or flour as feedstock.
3) Study the hydrolysate fermentation to ethanol
4) Study the possibility of a single stage, starch to ethanol process.
5) Perform the economic analysis based on the best case conditions.

Over the past two years, each task has been pursued and completed. This final report summarizes our experimental research work on all of the above areas and presents a final, promising economic analysis based on the data generated throughout the study.

2. DEVELOPMENT OF THE MEMBRANE BIOREACTOR

2.1. Equipment and Procedures

Bioreactor

A sketch of the bioreactor is shown in Figure 1. The reactor was fabricated as a 5 liter, jacketed glass vessel, approximately 33 cm high by 13 cm ID, with warm water circulating through the jacket to control the reaction temperature. The volume of the reactor was originally designed on the basis of continuous operation with 200 kg/m^3 starch feed and 95 % starch conversion. The reactor top was sealed through an O-ring by a flange cover having multiple ports for feed, product and sensors. To mix the starch slurry, two four-blade impellers with 45° pitches are mounted inside the vessel. This type of impeller produces mainly axial mixing which is known to be an effective method to circulate high density, particle slurries. The stirrers were driven by a Baldor DC motor, (Model AP7402, Baldor Electric Company, Fort Smith, AK), regulated by a variable speed controller. Because of the relatively low mixing speeds used for the experiments (400 rpm), the stirrer shaft was sealed from the reactor cover using a rubber O-ring.

Membrane Filters

Various membrane configurations were considered for the bioreactor. Based on our previous experience, we selected cross flow, polysulfone hollow fiber membrane filters from A/G Technology Co. (Needham, MA). Two types (stages) of filters were employed in the system. One type was a microfiltration (MF) membrane filter (CFP-2-E-3A cartridge, A/G Technology Co.) with 0.2 micron pore size and 1 mm fiber lumen

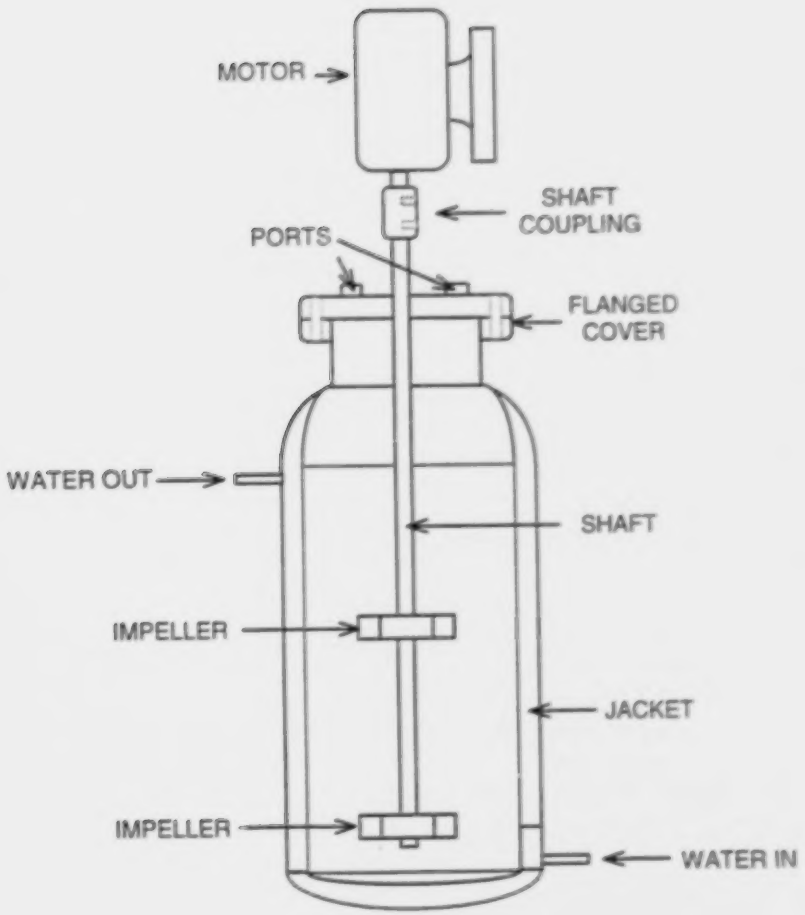


Figure 1. Schematic of the 5 Litre Bioreactor for Wheat Starch Hydrolysis.

diameter, installed just downstream of the bioreactor. The MF filer separates undissolved starch particles from the products. The other type was an ultrafiltration (UF) membrane filter (UFP-10-C-3A cartridge, A/G Technology Co.) with 10,000 NMWC and 0.5 mm fiber lumen diameter for retaining enzymes.

Since the membrane permeate flux decreases with operation time as a "gel" layer or a "fouling" layer develops on the membrane surface, these filters need to be periodically washed or cleaned. We installed a pair of filters in each of the two separation stages, such that when one filter is in service, the other can be cleaned in order to regain its full filtration capacity.

Membrane Bioreactor System

Figure 2 shows the schematics of our membrane bioreactor setup. Briefly, enzyme and starch mixtures were contained in separate, well mixed feed tanks. Each solution was fed to the bioreactor using peristaltic pumps. The hydrolysis slurry in the bioreactor was recirculated by a peristaltic pump through the microfiltration (MF) membrane for separation of starch granules (MF retentate) from sugars and enzymes (MF permeate). While the MF retentate returned to the bioreactor, its permeate was collected in a surge tank and pumped as feed to the next stage, an ultrafiltration (UF) membrane. The UF membrane further separated enzymes (UF retentate) which were returned to the bioreactor from sugars (UF permeate) which were collected as hydrolysis products (hydrolysates). Although only one MF and one UF membrane are shown in the schematics (for clarity), there were two MF and up to four UF membranes in the system. The MF membranes were connected through three way valves such that they could be used in parallel (for increasing filtration area) or in an alternating fashion (for cleaning).

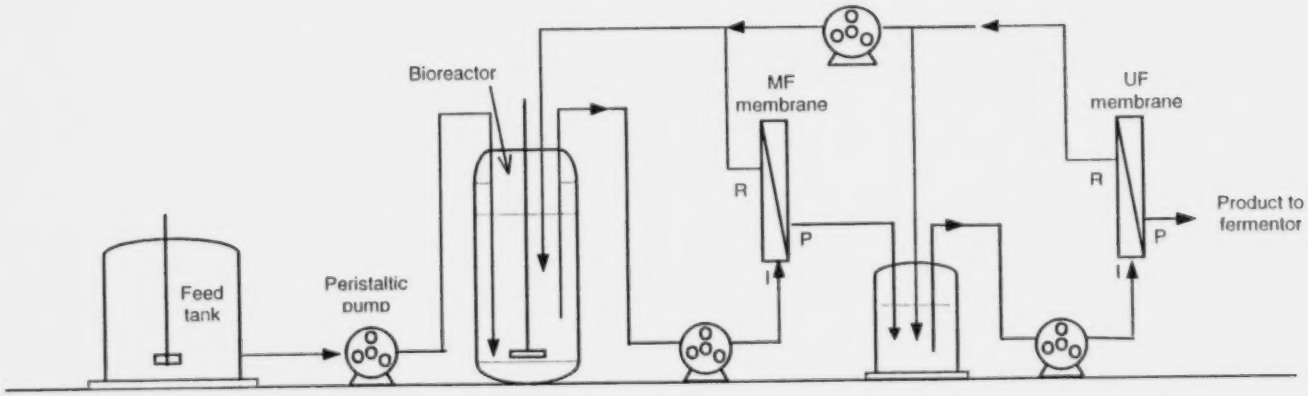


Figure 2. Schematic of the Starch Hydrolysis and Membrane Separation/Recycle System.

The four UF membranes were divided into two sets (two filters in each set) and were also connected through three way valves.

The entire membrane bioreactor system was anchored on a Dexion frame with back supporting panels. Whereas the motor for direct-drive stirring was firmly bolted on an aluminum panel, the large glass bioreactor was movable and was supported in an upright position by a special reactor stand, fabricated by the College of Engineering' Machine Shop. Before each experiment, the reactor stand could be lifted and mounted on the panel at a position such that the stirring shaft would always be in alignment when engaged with the motor, ensuring a smooth operation. After an experiment, the shaft could be disengaged from the motor, and the glass vessel in the stand was removed from the panel so the bioreactor could be cleaned and later placed into a steam sterilizer. Membrane filters were clamped on the panel and connected with other components via quick disconnect fittings; therefore, they could also be quickly and easily mounted or removed for cleaning. For sanitation purposes, all the valves, connectors and tubing in the system were selected using autoclavable materials.

To control the bioreactor temperature, a VWR model 1130A heated circulating water bath was used (VWR Canada, manufactured by PolyScience, Niles, IL). The water bath was capable of maintaining a stable temperature during hydrolysis and/or fermentation tests. The temperature difference between the bath and the reactor slurry was less than 0.5 °C.

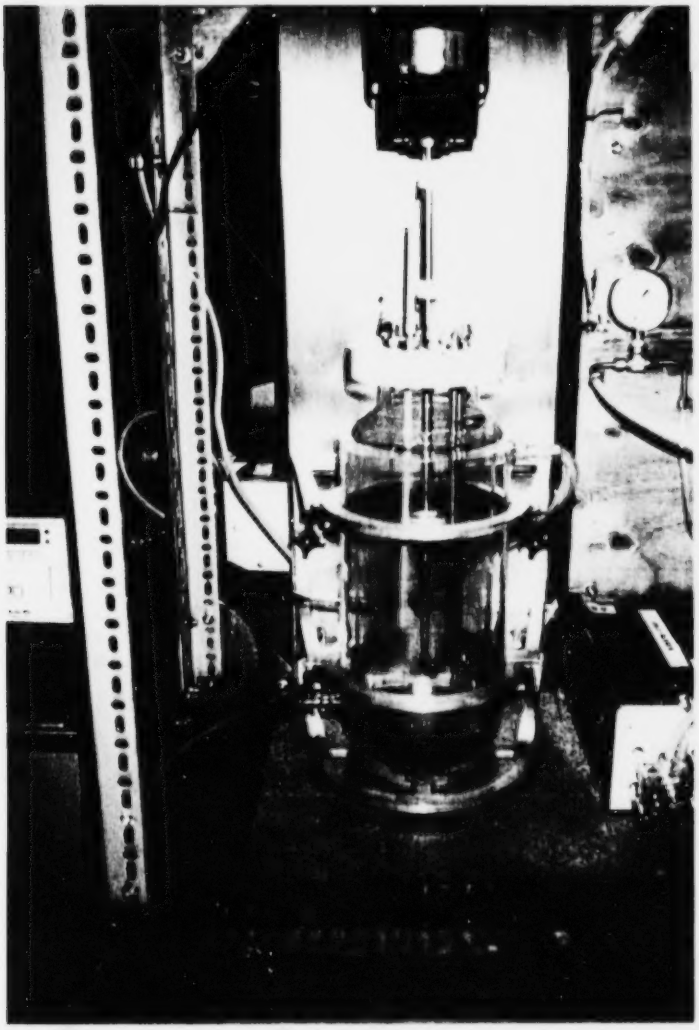
A conductivity type, liquid level controller (DYNA-SENSE Model 2220, by Scientific Instruments, Inc., Skokie, IL through Cole Palmer) was installed in the

bioreactor. The level controller regulated the feed pump on or off to keep the reaction volume constant during continuous hydrolysis.

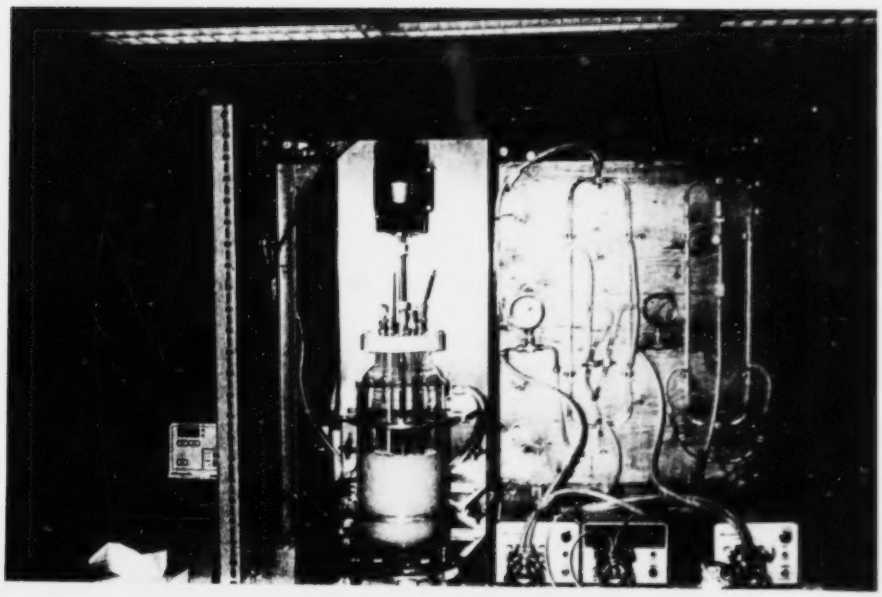
Two photographs are attached in this report, showing the actual arrangement of the system. The first one shows the empty bioreactor with its interior tubing and impellers. The second one is the front view of the system assembly during a real starch hydrolysis run: the motor and bioreactor are mounted on the left side metal panel; and the membrane section (including pressure gauges, filter cartridges and valves) are clamped on the right side wood panel. The slurry entering the microfitration (MF) filter was opaque, but it became clear when leaving the MF permeate side, demonstrating the total rejection of starch granules by the MF filter.

Sterilization Protocols

Proper protocols for sterilization were developed to ensure a contamination-free operation for starch hydrolysis. Whereas the feed starch, wheat or wheat flour were not sterilized but fed to the bioreactor as received; the reaction medium was sterilized by autoclaving at 121 °C for 20 min, as was the reverse osmosis water used for cleaning and washing the membrane filters. The empty bioreactor and all necessities for feeding (including beakers, tubing, feed tanks, etc.) were autoclaved at 110 °C for 20 min. The A/G membrane filter cartridges were sanitized with a 70 % ethanol solution as recommended by the manufacturer. The membrane sterilization was carried out on-line by circulating the ethanol solution through the entire membrane section (downstream of bioreactor) for one hour, followed by a thorough washing with sterilized clean water to remove ethanol residual. With such a protocol, the hydrolysis system was operated contamination free for over 100 continuous hours.



Photograph 1. Bioreactor



Photograph 2. The front view of the membrane bioreactor setup.

Protocols for Membrane Cleaning

The permeate flux of the hollow fiber, cross-flow membranes will decline with operation time, as large particles block the pores or a fouling layer develops on its surface, or because of polarization effects. Therefore, these membranes needed to be periodically cleaned to recover their permeability. We have examined different chemicals, including 0.5N NaCl, HNO_3, HCl and Terg-A-Zyme enzyme detergent, and have selected 0.2% Terg-A-Zyme solution (pH of 10-11) for routine cleaning because of its relatively mild condition and high cleaning efficiency. A typical cleaning procedure involved the following steps:

(1) Flushing: flush residual feed from cartridge with warm clean water;

(2) Cleaning: recirculate cleaning solution in "total recycle" (returning both retentate and permeate streams to the feed reservoir) for one hour, preferably leaving solution in cartridge overnight inside a 45 °C chamber;

(3) Rinsing: recirculating clean water in "total recycle" for 10 minutes, then drain solution, and repeat rinse two more times.

For MF filters, backflushing the cartridge with clean water at pressures up to 10 psig was also very effective.

2.2. Materials

Starch

Wheat starch, type A, was obtained from ADM/Ogilvie in Montreal, Que. It had a mean particle diameter of 21 microns (Textor et al. 1998).

The viscosity of starch solutions was determined at various concentrations using a cone/plate viscometer (Brookfield, model DV-II). Between 40 to 45 °C, and

concentrations from 30 to 200 mg/ml, the viscosities of these starch slurries ranged from 1 to 3 cp. These low values further underscore the advantages of not dissolving the starch particles as it is well known that concentrated starch solutions become very viscous. This information was used in designing pumping and mixing equipment.

Whole Wheat "flour"

Twenty-kilogram stone ground whole wheat flour was donated by Robin Hood Mills (Saskatoon, SK) in support of our project. The flour contains 14 wt% moisture, 13 % proteins, 2 % ash and 6 % others (fibers, vitamins), with the remaining 65 % being starch.

The wheat particle size distribution was measured using a Leeds and Northrup, Microtrac II Particle Size Analyzer. All meshes were found to be smaller than 700 microns. The mean diameter of the volume distribution was 298 microns, whereas over 80 % of the particles were larger than 88 microns and about 39 % larger than 350 microns. In comparison, the volume mean diameter of the ADM type A, wheat starch was only 21 microns. The whole wheat flour, therefore, is a much coarser product than wheat starch. To handle these coarse particles in the membrane bioreactor system, some modification had to be made

Processed Flour

An "untreated" flour, which was ground wheat with bran removed, was donated by Robin Hood Mills (Saskatoon, SK). The starch content in the flour is about 70 %.

Enzymes

Barley malt α-amylase (α-1,4 glucan glucanohydrolase, EC 3.2.1.1, type VIII-A) was purchased from Sigma – Aldrich Canada Ltd. (Sigma catalogue No A-2771).

Glucoamylase (amylogluosidase, or, 1, 4, α-D-glucan glucanohydrolase, EC 3.2.1.3) from Rhizopus mold was also obtained from Sigma (catalogue No A-7255).

Medium

The medium used for cold hydrolysis experiments contained 0.01 M of trihydrate sodium acetate ($C_2H_3O_2Na\,3H_2O$) and calcium chloride ($CaCl_2$) from 0.001 – 0.008 M depending on amylase concentration, in distilled deionized water, and its pH was adjusted by hydrochloric acid (HCl) prior to sterilization. The weak base, sodium acetate, paired with the strong acid, HCl, forms a buffer solution. The Ca^{2+} ion is vital for the maintenance of α-amylase stability

The medium for yeast fermentation studies used glucose or hydrolysate solutions as carbon sources, then supplemented with 10 g/L Bacto yeast extract, 1.4 g/L KH_2PO_4 and 0.33 g/L $MgSO_4$. The amount of yeast extract, KH_2PO_4 and $MgSO_4$ in the medium was based on 100 g/L glucose as carbon source. The pH was adjust to about 4.2 by HCl.

The medium for the simultaneous hydrolysis and fermentation works consisted of 2.73 g/L (0.02 M) $C_2H_3O_2Na\,3H_2O$, 0.46 g/L (0.004 M) $CaCl_2$; 10 g/L Bacto yeast extract, 1.4 g/L KH_2PO_4 and 0.33 g/L $MgSO_4$. The medium pH was also adjusted using HCl.

2.3. Analytical Methods

Starch

Starch granule concentration was measured using a Novaspec II (Pharmacia LKB Biochrom Ltd. UK) spectrophotometer at 600 nm after calibration against known weights of starch granules in the reaction medium.

Glucose

A portable blood glucose monitor (Model Accu-Chek Advantage, Boehringer Mannheim Canada) was used for quick determination of glucose concentration in solutions. For batch hydrolysis and fermentation runs, the slurry sample taken from the reactor was first filtered through a 0.2 micron syringe filters to remove starch granules and yeast cells, then the clear filtrate was used for glucose analyses. For continuous membrane filtration, the UF permeates were sampled for glucose measurements.

Chemical Oxygen Demand

Total sugar content in the hydrolysates (UF permeates) was determined by the chemical oxygen demand (COD) method using a system developed by HACH Company (Loveland, CO, USA). The COD results were reported as glucose in g/L based on a calibration with standard glucose solutions.

Enzyme

The activities of the Sigma barley malt α-amylase as well as that of various microbial α-amylase were measured according to procedure No. 577 of Sigma Diagnostics. The initial reaction rate, (d(OD)/min), at 30 °C and 405 nm was used to express the enzyme activity. Typical barely malt amylase activity was between 56-66 units per gram of solid.

Ethanol

The concentration of ethanol during fermentation was determined in a gas chromatograph (Model 5890, Hewlett-Packard) equipped with a flame ionization detector and a Supelco 30 m x 0.25 mm PTE-5 capillary column. GC analysis was conducted at

60 °C isothermally with 1-butanol as internal standard. Helium flowing at 1 ml/min was the carrier gas.

Yeast

The yeast cell concentration during fermentation was measured using a Novaspec II spectrophotometer at 600 nm and then compared with a calibration curve of absorbance against dry cell weight. The dry cell weight was obtained by first centrifuging fermentation broth three times at 5,000 rpm for 15 minutes with decanted liquid being replaced each time by RO water, followed by overnight drying at 65 °C in a vacuum oven.

3 COLD STARCH HYDROLYSIS

3.1. Batch Hydrolysis

Cold starch hydrolysis, i.e., degradation of starch below its gelatinization temperature (~ 60 °C), was first investigated in batch reactions. The test conditions were 45 °C, medium pH 4.5 and with barley malt amylase as liquefaction agents. The standard medium of pH 4.5 contained 0.01 M of sodium acetate and 0.001 M of calcium chloride in distilled deionized water.

The starting procedures for each experiment were as follows: first, the empty bioreactor was heated to the desired temperature by circulating warm water from the water bath through the reactor jacket. Next, the amylase powder was "dissolved" in a beaker with the medium and subsequently pumped into the bioreactor using a peristaltic pump. After the enzyme slurry temperature became stable, the desired amount of starch was suspended in fresh medium and pumped into the reactor. More medium was added until the slurry level reached the desired reaction volume, and this was considered as time zero for the experiment. The loading of starch lasted 5 minutes.

The stirring speed of the pitched impellers was set at 400 rpm. Simultaneous sampling of starch from different locations in the bioreactor gave reproducible results, which confirmed that the reaction slurry was well mixed under the selected mixing conditions.

Preliminary Batch Runs

The hydrolysis of insoluble starch granules in a 4 L bioreactor was compared in Figure 3 with that in a 25 ml small reactor for an initial concentration of starch, $S_0 = 30$

g/L and of barley malt amylase enzyme, E_0 = 8 g/L, respectively. We found that the starch degradation in the large bioreactor followed the same trend as in the small one, 95 % of the starch was hydrolyzed in a 24 h period. We could speculate that the mechanical forces from stirring were therefore not detrimental to the enzyme molecules and that there was no mass and/or heat transfer resistance in the scaled-up bioreactor. There were some discrepancies between 3 and 7 h, which probably was due to a low stirring speed in the large bioreactor during that period caused by motor/shaft problems; inadequate mixing would result in lower hydrolysis rates. After correction of the mechanical problem, the final starch concentrations from the two types of reactors were the same.

The batch mode was also tested for other initial starch concentrations of: S_0 = 10 and 100 g/L, respectively. As shown in Figure 4, the higher the feed starch concentrations, the lower conversion was the hydrolysis. This was also in agreement with what we have observed previously in small reactors using barley malt, bacterial and fungal amylases (Textor et al., 1998). We have attributed such behavior to enzyme activity inhibition by hydrolysis products.

Since it was desirable to work with high concentrations of starch feed in order to produce a concentrated glucose stream for later fermentation, we have studied the hydrolysis of S_0 = 100 g/L starch using two different enzyme concentrations (Figure 5). With 8 g/L barley malt amylase, about 70 % of insoluble starch granules were hydrolyzed in 30 h; further hydrolysis became much slower and the glucose concentration remained at 42 g/L. When using 30 g/L enzyme (Run 13), 70 % hydrolysis was achieved in 10 h; after 30 h, over 90 % of starch granules were dissolved whereas the glucose concentration increased to 75 g/L. Apparently, by employing more enzymes, (the

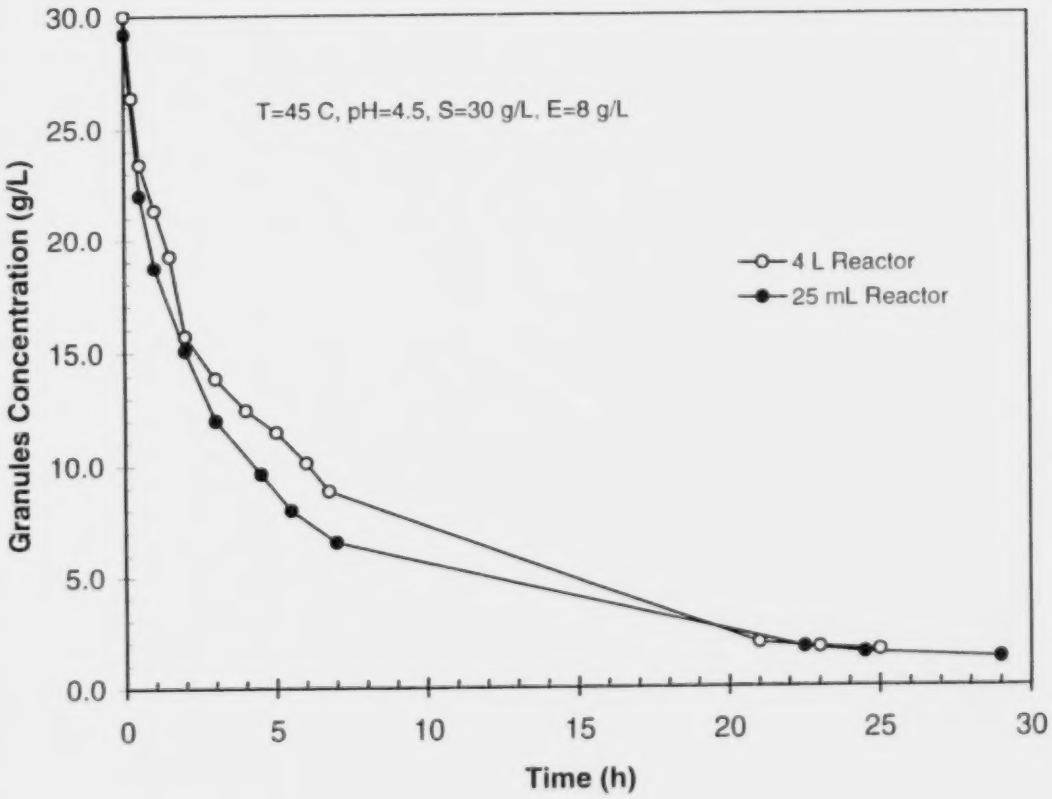


Figure 3. Comparison of Starch Hydrolysis between a 4 L and a 25 mL Batch Reactor.

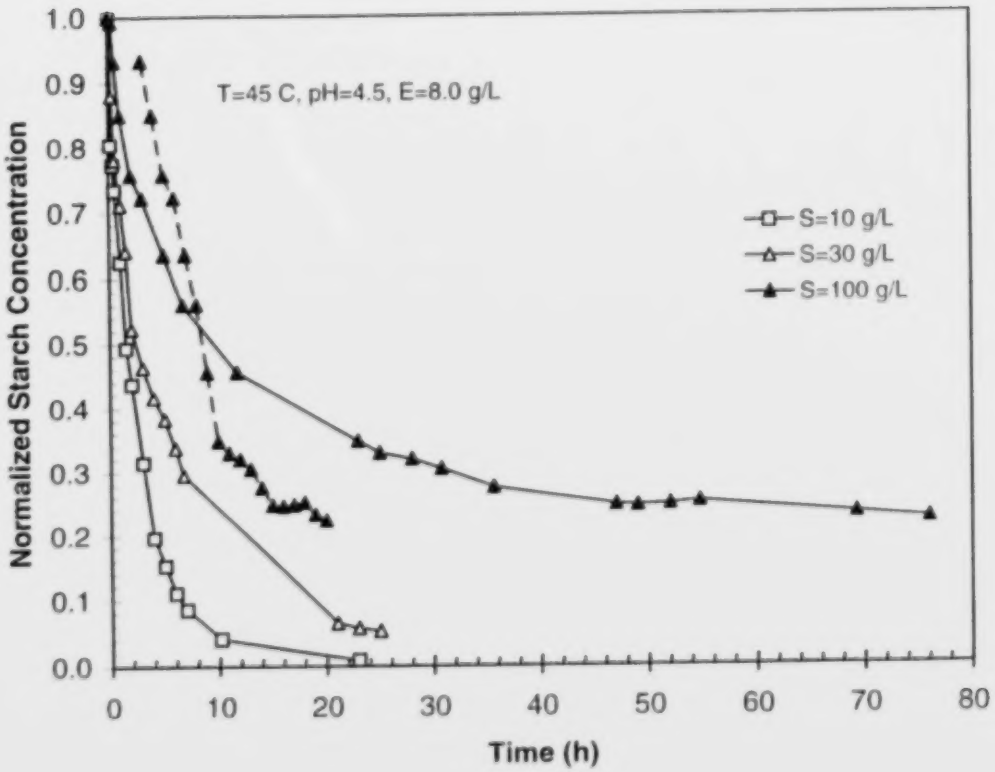


Figure 4. Starch Concentration Effects on Barley Enzyme Hydrolysis Rates.

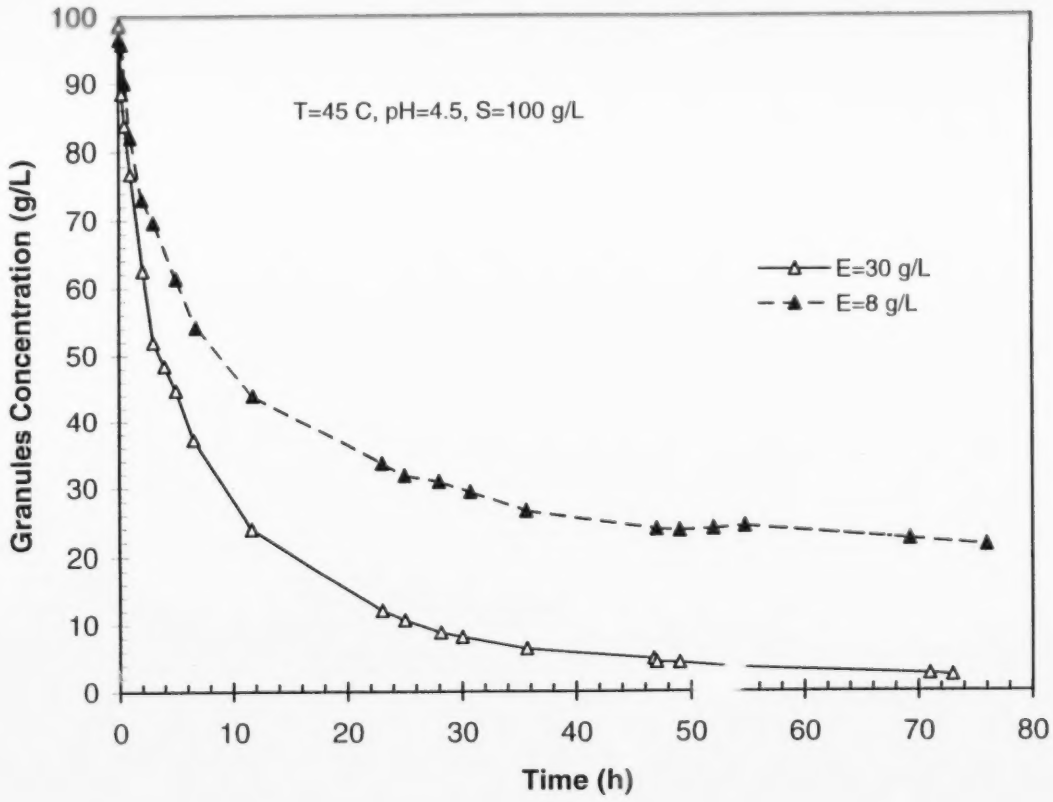


Figure 5. Barley Enzyme Concentration Effects on Starch Hydrolysis.

Catalyst) we can achieve higher starch conversion in a given time, resulting in higher concentrations of glucose in the product stream. The penalty is, however, higher enzyme concentrations increases the operating cost of the process. The activity of the enzyme at 30 g/L was assayed daily in a blank reactor (without starch). The initial activity was 1730 U/L (or 58 U/g) at 2.5 h, but it changed to 1380 U/L at 25 h and to 1000 U/L at 49 h. Such loss in activity has been reported by us before (Hill et al. 1997); and in the absence of substrate, was significant.

Through these preliminary batch runs, we proved that the bioreactor scale-up has met the process requirements. We also found the batch hydrolysis behavior in the 3 L bioreactor was in agreement with what we had determined before in smaller 25 ml reactors.

Run 15: Batch Test of the Membrane Bioreactor

Next, we further tested the system by linking the bioreactor to the membrane filters, but with both retentate and permeate streams returning to the bioreactor (total recycle). In test Run 15 at 45 °C, pH 4.5, starch S_0 = 100 g/L and barley amylase E_0= 30 g/L, the hydrolysis was initially allowed to proceed with no filtration until the starch concentration dropped below 40 g/L in the bioreactor (7.5 h). Then the reactor slurry was pumped through the membrane filters. The objective of this run was to observe if filtration would adversely affect the hydrolysis reaction. In Figure 6, the variation of insoluble starch concentration with time during run 15 is compared with run 13, which was conducted under the same conditions but without membrane filtration. We found there was little difference in starch hydrolysis between these two runs. Because of the high recirculation rate through the membranes filters, (about 900 ml/min for MF and 300

ml/min for UF, respectively), the residence time in the filters was so short that the filters can be viewed as part of the bioreactor. Because of the "concentration polarization effects" in an ultrafiltration (UF) filter, a fraction of the enzymes would always occupy a thin layer adjacent to membrane surface. However, considering the large quantity of enzyme used in our experiments and the relatively small membrane surface area, such hold-up loss of enzyme in our UF filter seems to have negligible effects on the hydrolysis rate. Shear on the enzyme when recirculating through hollow fiber membranes could cause its deactivation; however, under our operating conditions, it seems such an effect was not significant. The concentrations of glucose during these two runs are also plotted in Figure 6, and they are comparable, too. The final glucose concentration at 95 % conversion was about 75 g/L. The unaccounted part could be other oligosaccharides, which the glucose strip was unable to detect.

In run 15, a single pair of MF and UF filters were operated for about 70 h continuously, with no plugging problems. The permeate flow of the MF filter was monitored with time. The clean water flow rate of the MF filter was 20 ml/min immediately following a thorough cleaning procedure. After passing starch slurry through the filter, the permeate rate dropped below 10 ml/min within 30 minutes. It then remained relatively stable until the end of the test. The permeate rate of the UF filter was about 1 ml/min at the end, which was still large enough for continuous hydrolysis operation. Therefore, it was not necessary to clean the membrane everyday, which would simplify continuous operation substantially. Daily assay of the UF permeate detected no enzyme activity, confirming that the enzyme had been totally retained inside the bioreactor by the UF filter.

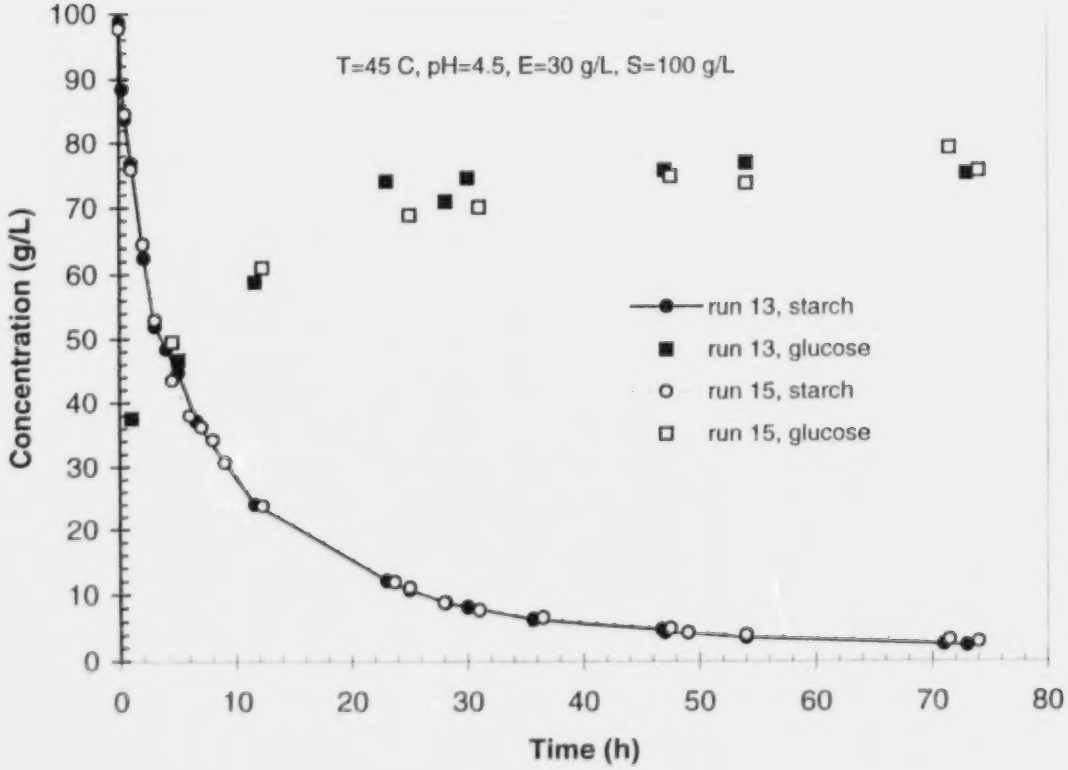


Figure 6. Comparison of Starch Hydrolysis between a Bioreactor (Run 13) and a Membrane Bioreactor (Run 15).

3.2. Continuous Flow Hydrolysis

The objective of the continuous hydrolysis runs was to establish the operating conditions for the effective production of a continuous stream of hydrolysates containing high concentrations of glucose and oligosaccharides suitable for ethanol fermentation. A total of six runs were conducted. The effects of hydrolysis temperature, medium pH and Ca^{2+} ion concentration in the medium were investigated. Whereas all the data has been reported in our interim progress reports, detailed results of three key tests are presented in this section.

Run 16

The first continuous hydrolysis test, Run 16, was at 45 °C, medium pH 4.5. The run started in batch mode using 30 g/L starch and 8 g/L enzyme initially inside the bioreactor (slurry volume of 3 L). After 7 h, when the starch hydrolysis rate in the bioreactor (mass per hour) approached the intended feed rate, continuous operation was commenced. First, the circulation pumps to the membrane filter were turned on. After the operation of both MF and UF filters became stable, the UF permeate was withdrawn from the system and collected in a product tank. Then the feed pump was turned on, sending a feed of 30 g/L starch slurry at 1 ml/min from the feed tank, which was continuously stirred, to the bioreactor. The time variation of insoluble starch concentration in the bioreactor during Run 16 is shown in Figure 7 (closed symbols). The starch concentration was 7 g/L inside the bioreactor when the continuous feeding started at 7 h on stream. It gradually increased with time and at 73 h it became over 20 g/l when the run was terminated. In the mean time, the glucose concentration decreased from 17 to 10 g/L. An assay at the end of the run detected a near zero enzyme activity in

the bioreactor. Because of enzyme inactivation, the hydrolysis rate of starch became less than its feed rate, this caused accumulation of starch in the bioreactor and prevented continuous flow operation to attain a steady state.

We then investigated different methods for improving enzyme stability. Lower temperatures and less acidic pH alleviated the enzyme inactivation rate, however, they also reduced the hydrolysis rate significantly.

Run 19

Ca^{2+} ions have been known to greatly enhance the thermostability of α-amylase. According to the literature, a ratio of over 10 Ca^{2+} ions per enzyme molecule is required for stabilization. Because of the high enzyme concentration used in our experiments, we decided to double the Ca^{2+} content in our medium from 0.001 to 0.002 M.

In run 19 at 45 °C, pH 4.5 and Ca^{2+} of 0.002 M in the medium, the results are plotted in Figure 7 (open symbols), together with that from run 16 to evaluate the effects of medium Ca^{2+} concentration. As shown in Figure 7, after switching to continuous hydrolysis in run 19 (Ca^{2+} = 0.002 M), the insoluble starch concentration still increased with time, but at a lower rate than in run 16 (Ca^{2+} = 0.001 M), implying that doubling the calcium ion concentration was favorable to stabilization. The glucose concentration during the continuous period went from 17 to 13 g/L. The assay of the enzyme activity at 77 h found 80 U/L, down from 1700 – 2000 U/L (56 – 66 U/g) which was typical for a fresh enzyme sample. Therefore, even by doubling Ca^{2+} ion concentration, the enzyme inactivation was still significant.

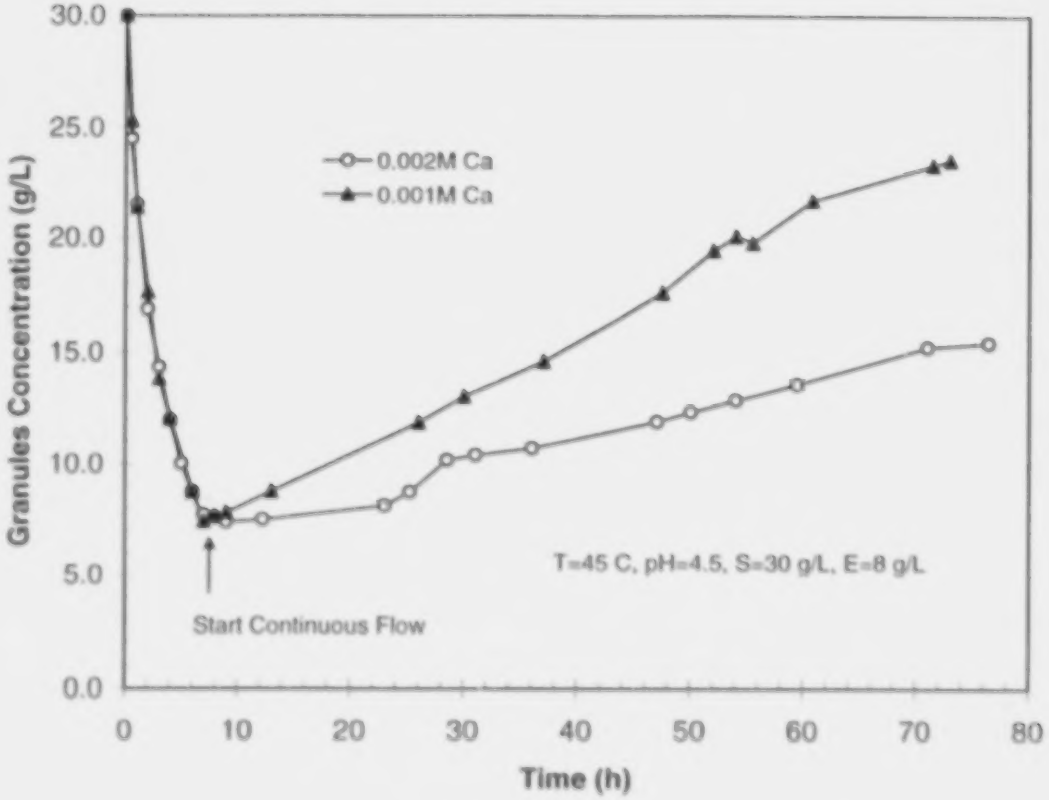


Figure 7. Effects of Medium Calcium Concentration on Enzyme Stability.

Run 22

Run 22 used a concentrated, 100 g/L or 10 wt%, starch feed. This feed could produce a hydrolysate with a high sugar concentration needed for down stream ethanol fermentation. To test long term continuous operation, we planned to add fresh enzyme intermittently during the run to compensate enzyme inactivation. This run lasted 274 h and is described in detail here.

The hydrolysis was studied at 40 °C and pH 4.5. Lowering the temperature from 45 to 40 °C could improve the enzyme stability but not significantly lower the activity. About 100 g/L starch and 30 g/L barley malt α-amylase were first loaded in the bioreactor (slurry volume of 3 Liters). For such a high enzyme concentration, the Ca^{2+} ion concentration in the medium was correspondingly increased to 0.008 M in order to preserve enzyme stability. After 24 h of batch reaction, about 90 % of the starch was dissolved and the glucose concentration had reached 70 g/L. At this point, continuous operation was commenced.

During this run, a liquid level controller had been installed in the bioreactor and it controlled the feed pump to keep the reaction volume constant. Since the feed flow rate during the continuous run depended on the product withdrawal rate (UF permeate rate), in order to minimize the starch accumulation in the bioreactor, the average UF permeate rate was set at about 0.5 ml/min by regulating the UF transmembrane pressure. Consequently, the average feed flow rate was about 0.5 ml/min, with the feed starch concentration set at 100 g/L.

Figure 8 shows the concentration variation of starch granules in the bioreactor and of the total sugar in the products. The total sugar (glucose, maltose and other

oligosaccharides) in the products was determined by the COD method in a HACH COD reactor and was calibrated on a glucose basis. During the first 120 h of continuous hydrolysis, the total sugar concentration in the hydrolysate was over 80 g/L, and it reached 96 g/L around 100 h on stream. Even at a reduced feed rate, not all of the starch fed to the reactor was hydrolyzed and the unreacted granules accumulated in the bioreactor. To reduce starch accumulation, the starch feed was stopped and the reaction was switched to batch mode from 150 to 164 h on stream. The remaining enzyme activity at this time was rather low; an assay of enzyme activity found to be only 10 % of the initial activity in the bioreactor. To continue the operation, 27 g of barley enzyme powder was added to the bioreactor between 167 - 195 h. After that, the sugar concentration in the product increased and was maintained at 90 g/L from 174 until 214 h on stream. At 242 h, another 30 g of enzyme was added and the operation time was extended to 274 h. At this point, the enzyme activity was completely gone and the run was terminated.

As the starch accumulated in the bioreactor, the membrane permeability deteriorated. Since we had anticipated this problem and installed two sets of membranes in parallel, when the permeate rate became too low, the clogged membrane was replaced by a clean one through a simple valve switching and the permeate rate was restored. The clogged membrane was then removed for off line cleaning. This arrangement permitted long term operation of the membrane bioreactor.

In conclusion, continuous flow hydrolysis exposed the problem of enzyme inactivation. As a result, a steady concentration of starch could not be maintained in the bioreactor. Our solution was to allow its concentration to vary within a certain range, and

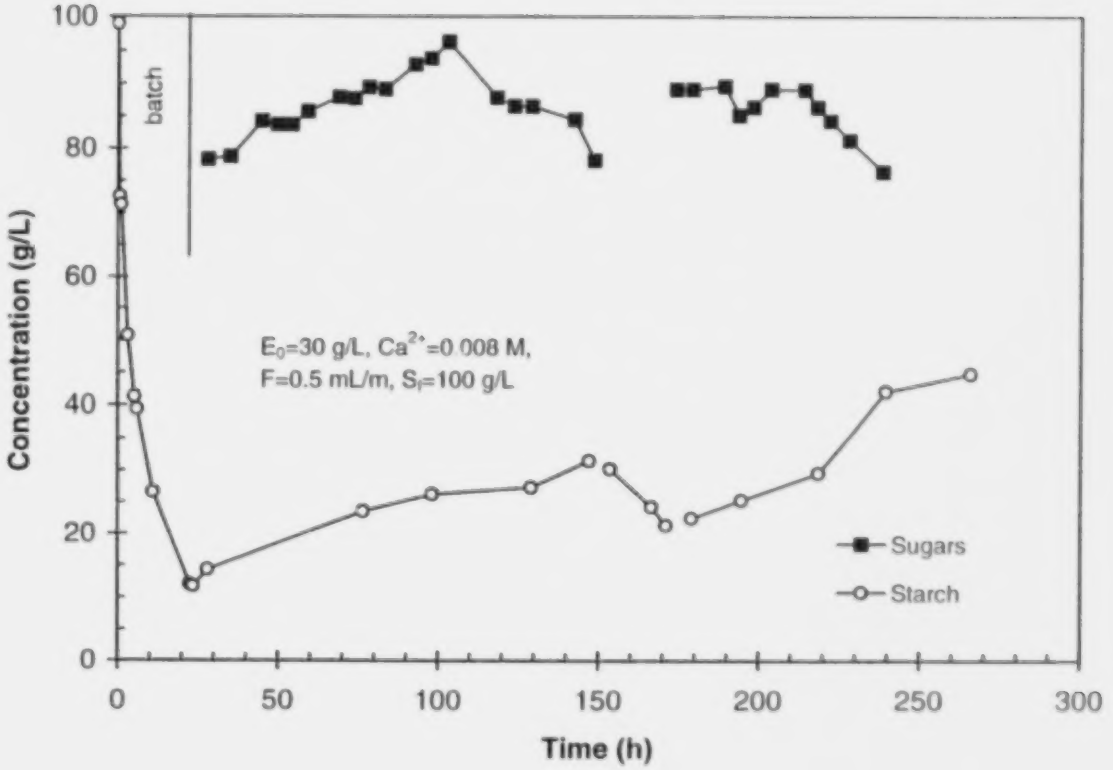


Figure 8. Starch Hydrolysis at pH 4.5 and 40 °C in a Continuously Well Mixed Membrane Bioreactor (Run 22).

to supplement fresh enzyme periodically when starch accumulated beyond 30 g/L causing deterioration in membrane filtration. This strategy had been partly successful in Run 22. The belief is that any further success of prolonged continuous hydrolysis depends on the improvement in enzyme stability.

3.3. Enzyme Studies

As we tried to improve the stability of the barley malt amylase, we have investigated the effects of temperature and the product concentration on enzyme activity. We have also explored the possibility of using thermal stable bacterial α-amylase for cold starch hydrolysis. The results on these enzyme studies are reported in this section.

Temperature Effects on Decay of α-Amylase Activity.

Figure 9 shows data from three experiments measuring the deactivation of barley α-amylase, one experiment at 55 °C and two experiments at 45 °C. In this figure, the enzyme activity is made relative by dividing by the activity at time zero in all cases. The scatter in data during the 45 °C tests was primarily due to problems with temperature control (± 2 °C) which did not occur in the earlier 55 °C test (± 0.5 °C). Nevertheless, the trends are clear, the enzyme degrades much faster at 55 °C compared to 45 °C and the degradation is linear in time. The loss of activity was 1.1 percent per hour at 45 °C and 3.2 percent per hour at 55 °C. Although the data does not fit an exponential decay model, linear time decay does agree with observations plotted earlier by Tkachuk and Kruger (1974) using α-amylase at temperatures up to 60 °C. When modeling hydrolysis kinetics at 45 °C, the linear decrease in enzyme activity with time (t in hours) must then be incorporated into the predictions by:

$$E/E_0 = 1 - 0.011 \times t \qquad (1)$$

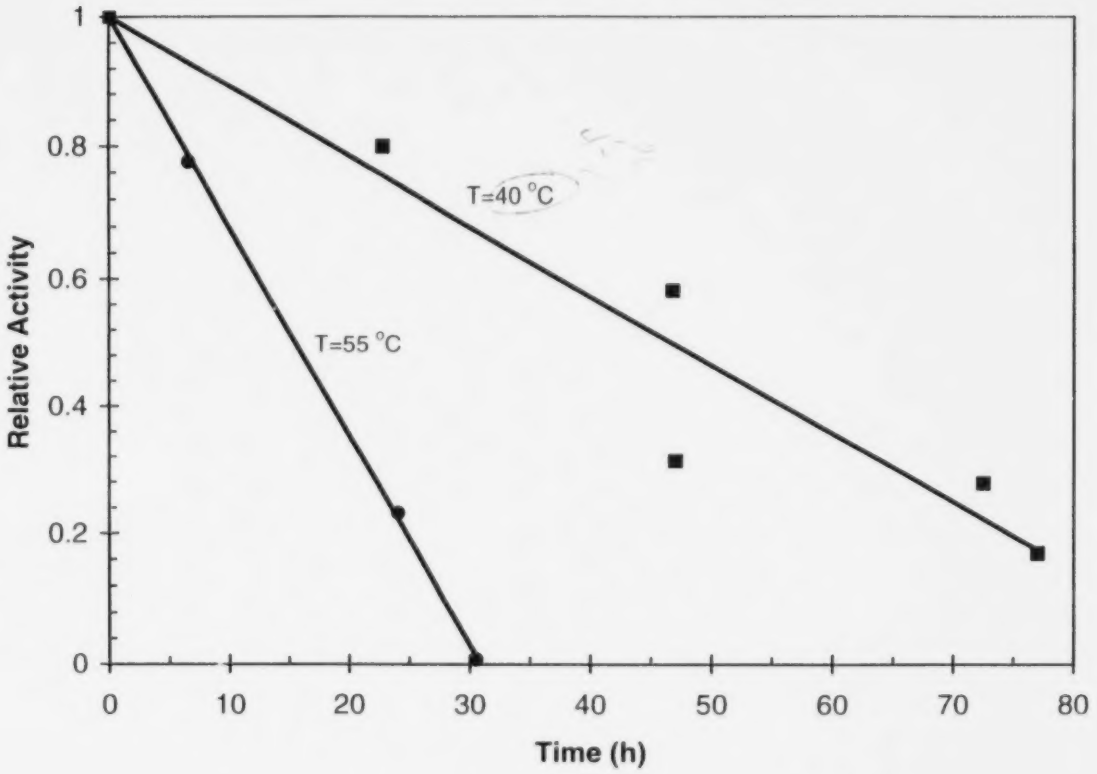


Figure 9. Temperature Effects on the Decay of alpha-Amylase Activity.

Product Inhibition Effects

The effects of glucose and maltose concentration on the initial reaction rates were studied by first adding enzyme and sugar to a well-mixed reactor for 15 minutes before adding starch granules. The results are shown in Figure 10. Both of these starch hydrolysis products are seen to affect the hydrolysis rates in the same manner. Furthermore, both the competitive and non-competitive inhibition models could not be made to fit this data closely. Instead, a simple exponential inhibition function was fit to the data and the model (solid line) is seen to be quite close to the data with an average deviation of about 10 % between the predicted and experimentally measured rates. The equation to predict the effect of product (glucose and/or maltose) concentration (P in g/L) on the granule hydrolysis rate is then given by:

$$\frac{dS}{dt} = \left(\frac{dS}{dt}\right)_{P=0} \times \exp(-0.0083 * P) \qquad (2)$$

It is not clear that the cause of inhibition is directly related to the product molecules binding to the active or other sites on the α-amylase enzyme, since both competitive and non-competitive inhibition models failed to predict this inhibition behavior. It could be an osmotic effect caused by the very high sugar concentrations reducing the water activity in the vicinity of the enzyme-starch complex.

Textor et al. (1998) have proposed an enzyme reaction rate model (i.e. dS/dt at P = 0) consisting of two parts. Initially, easily hydrolyzed components of the granules are completely reacted within a few minutes (first order rate constant estimated to be 40 h^{-1}). Hydrolysis was then followed by much slower Michaelis-Menten kinetics with the enzyme and starch granule concentrations changing roles in the theoretical model of soluble substrate - enzyme catalyzed reactions. Both Equations 1 and 2 are now valuable

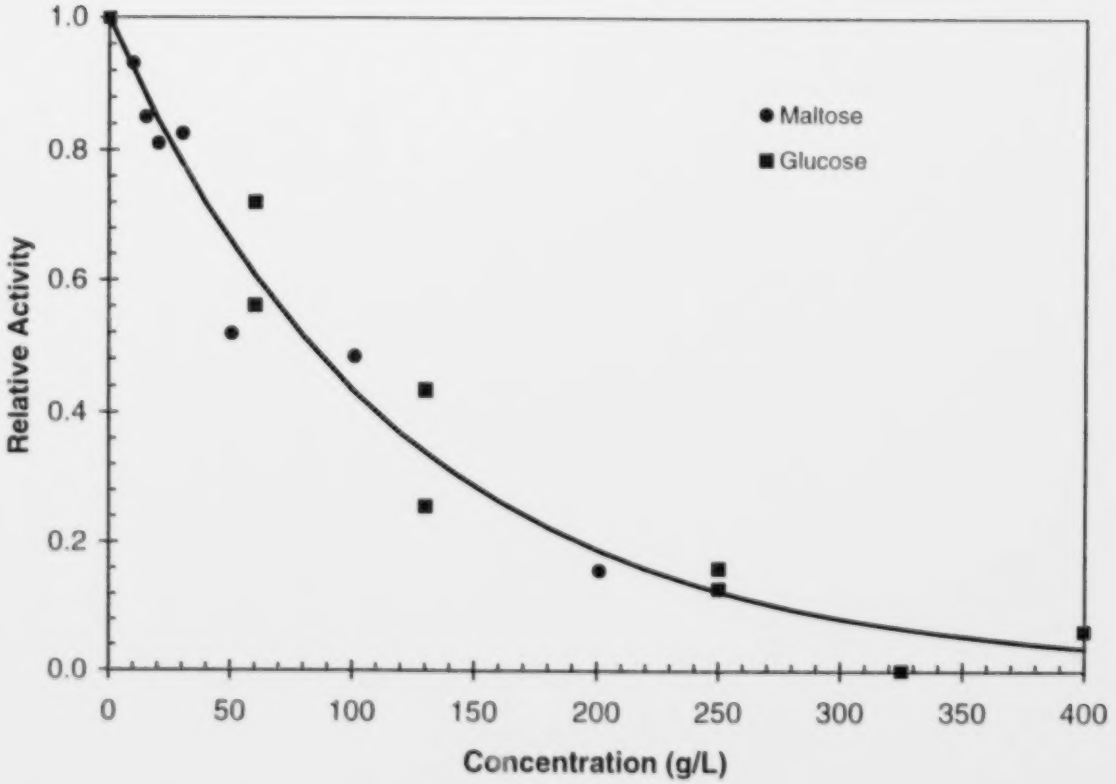


Figure 10. Exponential Inhibition Effects of Hydrolysis Products on the Relative Activity of Barley Amylase.

additional information needed for long-term predictions of the behavior of an industrial reactor. In particular, it can be seen that due to the very high inhibiting effect predicted by Equation 2, when the hydrolysis product concentration reaches 100 g/L (sugar concentrations needed for fermentation ethanol), the operation of a continuous, well-mixed reactor would not be a favorable design strategy due to a drop of about 50 % in enzyme activity. This suggests that an enzyme starch hydrolysis reactor might best be operated in either batch or fed-batch mode, or for continuous operation, a plug-flow tubular reactor should be considered.

Heat Stable *B. licheniformis* Amylases

Most industrial processes of fuel ethanol fermentation (gasohol) use thermostable microbial amylases from *Bacillus licheniformis* for starch liquefaction at high temperatures (90-140 °C). To determine the prospect of using the *B. licheniformis* amylase for cold starch hydrolysis, we have studied a total of four *B. licheniformis* amylase samples from different sources. They were (1) heat stable *B. licheniformis* amylase in lyophilized powder form (this species was heat stable up to 140 °C) purchased from Sigma (Sigma catalogue No A 4551); (2) Type XII-A bacterial amylase from *B. licheniformis* in aqueous solution from Sigma (Sigma catalogue No A 3403), which was stable at temperatures as high as 90 °C; (3) aqueous HIGH T 2X α–amylase donated by Alltech, Inc. (Nicholasville, KY) and (4) liquid thermostable α–amylase, Maxamyl®, donated by Gist-Brocades (Charlotte NC). From our analyses, we found the amylase species in the last two commercial samples were the same as in the aqueous Sigma sample.

These thermostable, *B. l.* α-amylases were found to be time-stable at 40 °C. For example, at pH 6.0, the Sigma bacterial enzymes lost only 20 % of their initial activities after 144 hours of operation. Barley amylase usually completely inactivated after about 100 h at this temperature.

We have tested these *B. licheniformis* α–amylases for starch hydrolysis at 40 °C in a small batch reactor. Different optimal pHs were used for different species: pH 5.5 for the powdered form *B. l.* amylase from Sigma, whereas, pH 6.0 for both HT 2X and Maxamyl® enzymes. The results are summarized in Figure 11, along with the results of barley malt amylase. For hydrolyzing a starch slurry of 100 g/L, a typical barley malt amylase usage was 30 g/L, which was equivalent to about 2,000 U/L (activity unit per liter). In Figure 11, the enzyme usage was about 10,000 U/L for both powder form Sigma *B. l.* α-amylase as well as Alltech HT 2X amylase, and was 13,600 U/L for the Maxamyl® enzyme. Over 24 hour periods, 90 % of the starch was dissolved when using barley amylase, but only about 70-75 % of the starch was dissolved when using the different heat stable *B. l.* α-amylases although their enzyme activity levels were 5 -7 times higher than the barley enzyme. Less microbial enzyme gave even poorer starch dissolution as tested in other (not reported) runs.

The Alltech HT 2X enzyme was also tested for two days in the membrane bioreactor at 40 °C, pH 6.0 and an enzyme concentration of 10,000 U/L for a starch slurry of 130 g/L. About 84 g/L starch was hydrolyzed after 47 h. When using barley amylase, two times more starch had been hydrolyzed over the same period (completing two cycles in Run 24). Again, we found the heat stable amylase enzymes were less effective.

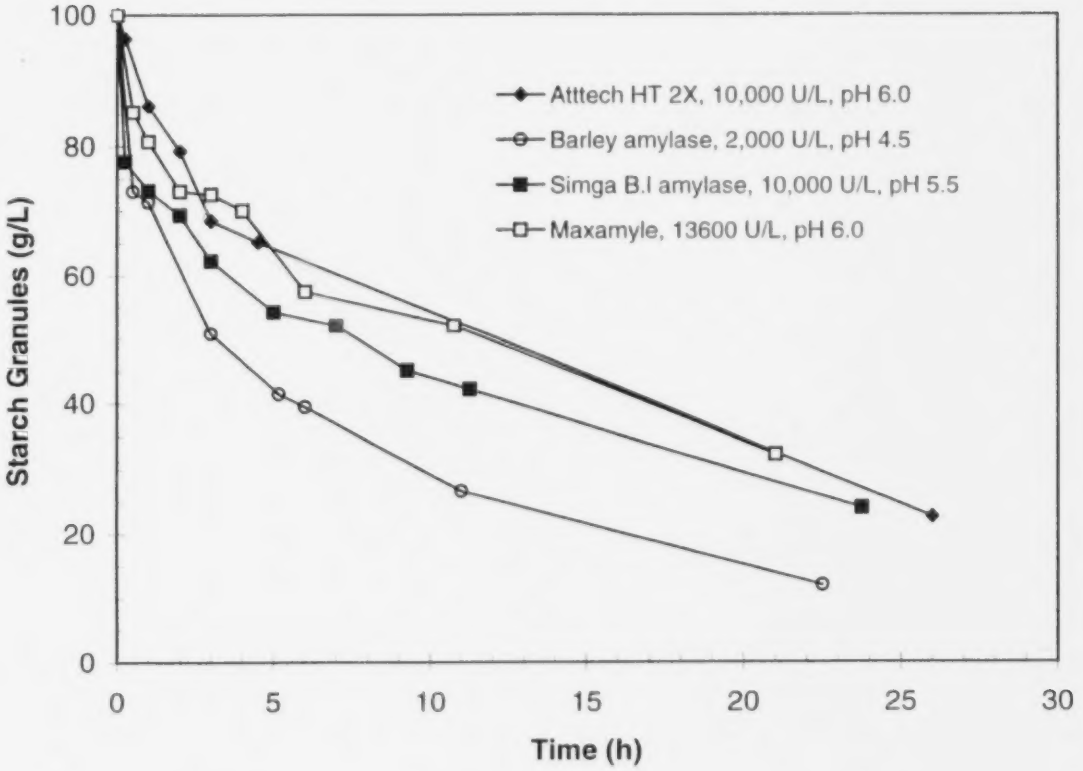


Figure 11. Comparison of Activities of *B. Licheniformis* Amylases and Barley Amalyse towards Hydrolysis of Starch Granules at 40 °C.

The optimal temperature for *B. l.* amylase such as Alltech HT 2X is around 90 °C. At 40 °C, the remaining activity is reduced to merely 25 % (Alltech product literature). Although the activity can be maintained at 40 °C, pH 6.0 over a long time, it seems the *B. licheniformis* amylase was much less effective in attacking insoluble starch granules at low temperature than towards soluble starch at high temperature. We have concluded, therefore, that for the cold starch hydrolysis, the barley malt amylase, despite being less stable, is a better choice than the heat stable microbial amylases. We therefore continued to use the barley malt amylase as the liquefaction enzyme in the next stage of the project, hydrolysis in a sequential fed batch operation.

3.4. Sequential Fed-Batch Hydrolysis

Since high product (glucose, maltose) concentration resulted in severe inhibition of amylase activity during continuous, well-mixed reactor operation, we developed a sequential fed-batch process for cold starch hydrolysis. In this process, the hydrolysis and filtration operations were separated and repeated in sequence:

(1) Hydrolysis Step: The starch slurry was loaded into the bioreactor and was hydrolyzed in a batch process until the total sugar concentration became high enough (90-100 g/L) for ethanol fermentation, then the stirring in the bioreactor was stopped and the filtration was initiated.

(2) Filtration Step: The reaction mixture was separated by membrane filtration to remove sugars and to return the enzymes and undissolved starch granules back to the bioreactor. The filtration step was stopped when 80% of the liquid was removed. Fresh starch slurry was then added to the remaining content of the bioreactor, and the next cycle

of hydrolysis begun. The clogged membranes were cleaned during batch hydrolysis periods.

The advantage of this mode of operation over continuous flow in a well-mixed reactor is that at the beginning of each fed-batch reaction step, the substrate concentration was high and the product concentration was low, so the product inhibition on the enzyme activity was greatly reduced.

Run 23: Sequential Fed-batch Hydrolysis

We first tested the sequential fed-batch operation in Run 23 at 40 °C and pH 4.5, with 0.008 M of Ca^{2+} ion in the medium. The barley malt α-amylase usage for the first batch was 30 g/L and no additional enzyme was added during the subsequent batches. The starch concentration in the first batch was 130 g/L; the higher feed concentration (than previously used) allowed the production of sugar concentrations up to 90 - 100 g/L at less than complete conversion (which would require very long times). A total of four cycles were completed over 170 h in Run 23. Figure 12 illustrates the decrease in starch concentration and the increase in glucose concentration with time in each of the four batch steps. The glucose concentration in the hydrolysates was also analyzed at the end of each filtration step, and the total sugar content in the final product was determined by the COD method.

The first batch hydrolysis took 12 h to hydrolyze about 90 g/L of starch, the second lasted 19.5 h, then 34 for the third and 62 h for the last cycle. The duration of each hydrolysis and subsequent filtration times are listed in Table 1. Longer hydrolysis times for each subsequent batch were required because of enzyme inactivation with time.

as indicated by the enzyme activity assay data in Table 2. It seems little enzyme activity was left after 100 h (4th cycle).

The filtration period lasted between 11 to 16 h. On average, 2.4 L of ultrafiltration permeate out of 3.0 L of slurry was collected from each filtration. The total sugar concentrations in the three batches of products ranged from 94 to 99 g/L (based on glucose), which is high enough for ethanol fermentation. The average glucose and total sugar concentrations in each batch of product are compared in Figure 12???. Glucose concentration decreased from the first batch to the next one, while the total sugar was similar for all three batches. It is possible that with time, hydrolysis proceeded to intermediate hydrolysis products (oligosaccharides) rather than the complete product (glucose), probably because of enzyme inactivation.

The three sequential batch hydrolysis-membrane filtration cycles in Run 23 lasted 108 h, and a total of 7,170 g of hydrolysate were produced with an average total sugar concentration of 95 g/L. In comparison, under continuous flow operation of Run 22, about 7,210 g of hydrolysate with an average total sugar concentration of 80 g/L were collected over a 270 h period and with 60 % more enzyme consumed. The advantage of sequential fed-batch operation for the cold starch hydrolysis is therefore very significant!

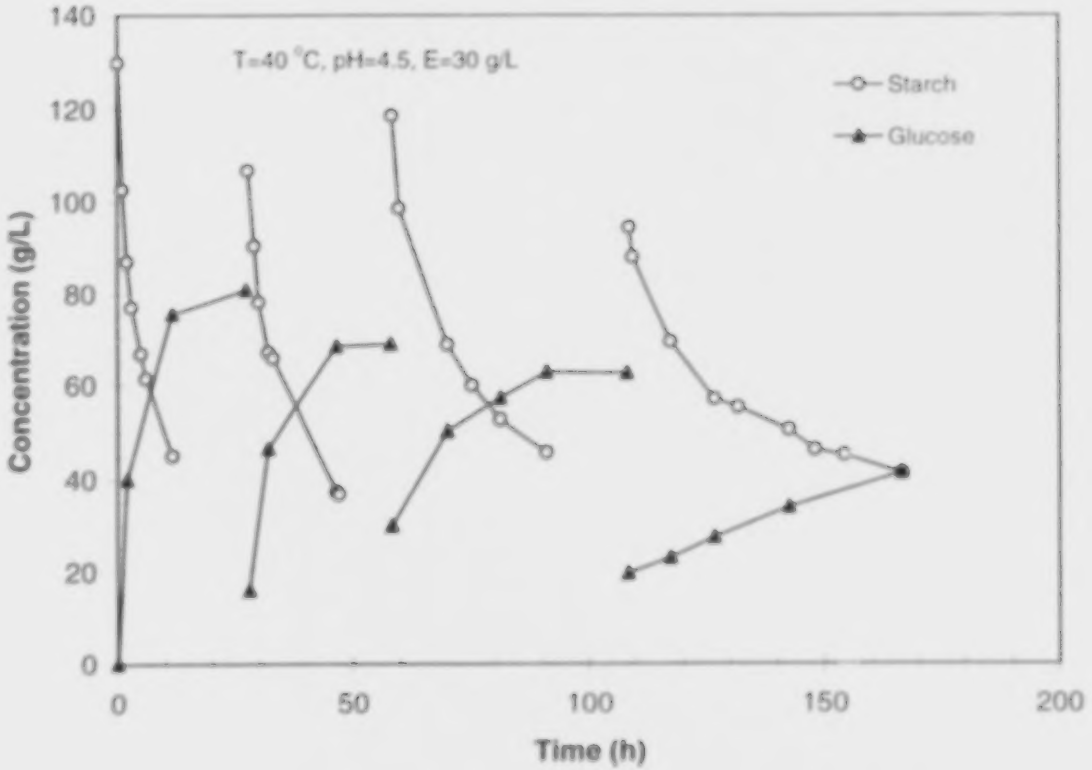


Figure 12. Starch Hydrolysis at pH 4.5 and 40 °C in a Sequential Fed-Batch Process (Run 23).

Table 1: Duration (in hour) of Each Step in Runs 23, 24 and 25.

Batch #	Operation	Duration of Operation, h		
		Run 23	Run 24	Run 25
1	reaction	12	12	15
	filtration	15.5	3	3.5
2	reaction	19.5	17.5	30
	filtration	11	4	4.3
3	reaction	34	29.5	56
	filtration	16	4.4	4
4	reaction	62	36.5	n.a.
	filtration	10.5	3.4	n.a.
Total time		184	113	114

n.a.: not applied

Table 2: Activity of Barley Amylase at Different Times during Run 23.

Times on Stream, h		Activity, %
0	(beginning of batch 1)	100
28	(beginning of batch 2)	76
58	(beginning of batch 3)	18
91	(end of batch 3)	5
117	(10 h into batch 4)	0

Run 24: Reducing Filtration Time

The efficiency of the sequential fed-batch operation was further improved by a reduction in filtration time. Since the ultrafiltration was the slowest step, we installed two more UF membranes in the system after Run 23, so that a total of four UF membranes were connected in parallel to increase the ultrafiltration rate.

In Run 24 (Figure 13), the average filtration times were reduced to 4 h from 11 - 16 h in Run 23. Shorter filtration time also shortens the batch reaction times for batches 2 and 3 (Table 1), since the enzyme was more active at earlier times on stream. As a result, the first three cycles in Run 24 were completed within 72 h, compared to 108 h that it took in Run 23. This was a saving of 36 hours. The hydrolysis became slower in batch 4, and an assay of the slurry at 109 h found no significant enzyme activity. Therefore, this run was terminated at 113 h after finishing the filtration for the fourth cycle.

The amount of liquid products collected from each cycle and their total sugar concentrations were comparable to what had been obtained in Run 23. The comparison of enzyme efficiency, or productivity, will be discussed after Run 25.

Run 25: Effect of Enzyme Loading

The effects of enzyme loading on the productivity have also been studied. In Run 25, 15 g/L of barley malt amylase, i.e., only half of the previous enzyme loading, was used in the hydrolysis. The Ca^{2+} ion concentration was lowered to 0.004 M, whereas other conditions were the same as in Runs 23 and 24. Changes of starch and glucose concentration with time during this run are plotted in Figure 14, and the time of duration for each step is listed in Table 1.

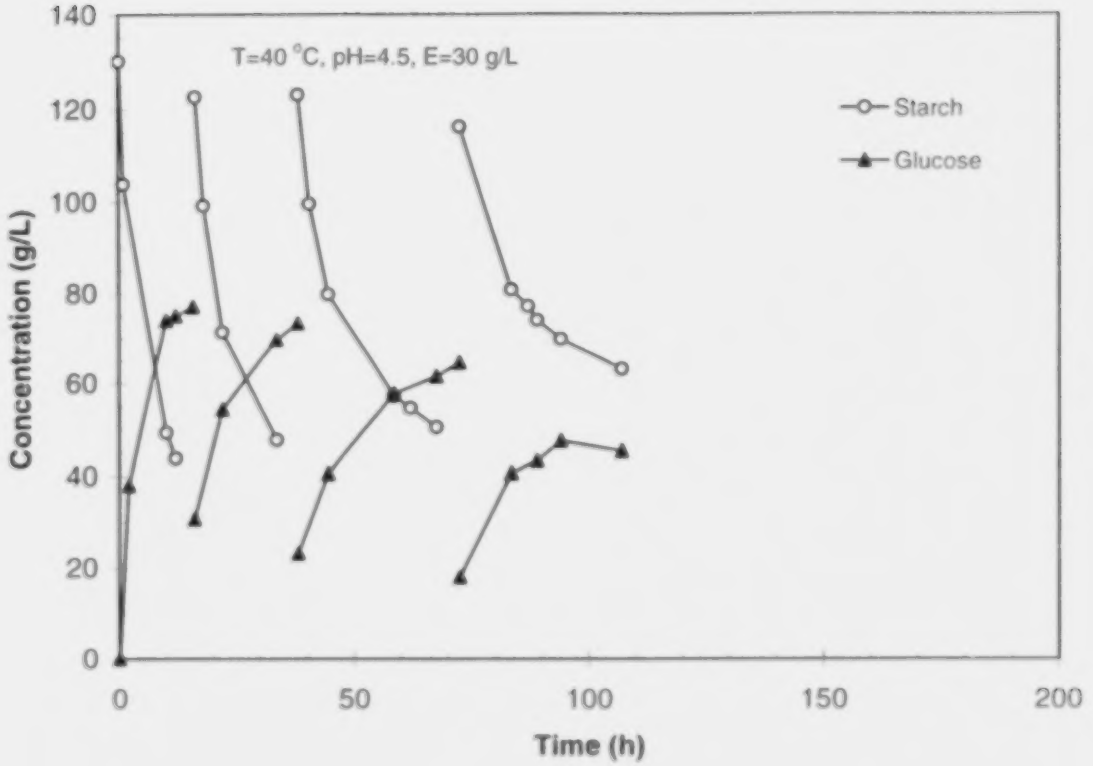


Figure 13. Starch Hydrolysis at pH 4.5 and 40 °C in a Sequential Fed-Batch Process with Reduced Filtration Times (Run 24).

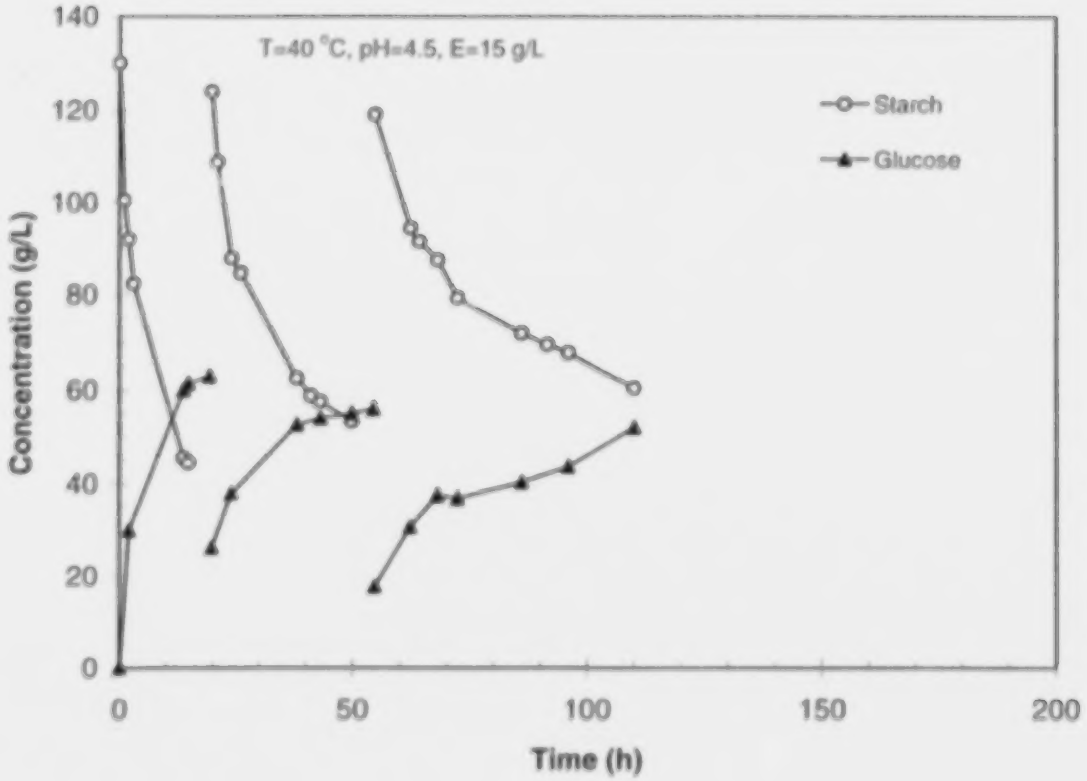


Figure 14. Starch Hydrolysis at pH 4.5 and 40 °C in a Sequential Fed-Batch Process with Half of the Enzyme Loading (Run 25).

The first batch of Run 25 took 15 h, about 3 h longer to hydrolyze a similar amount of starch as that of Run 24. However, since the enzyme loading was reduced to half, this represented a big improvement in enzyme productivity. The reaction time in the subsequent second and third batches were now much longer, 30 and 56 h, respectively (Figure 14). One explanation was that some of the enzymes were trapped on the membrane during filtration and lost for further hydrolysis. This loss would become more significant if less enzyme was added to the reactor. In addition, the products obtained after Run 25 had similar total sugars but lower glucose concentration than those of Run 24, so the hydrolysis was also less complete. The average glucose concentration in the three hydrolysates batches were 62, 54 and 52, respectively; the total sugar was about 105 g/L in the first two batches but 82 g/L in the third batch. As found in the previous runs, the hydrolysis proceeded very slowly after 100 h; therefore Run 25 was terminated at 114 h after only three cycles.

The enzyme productivity, in terms of grams of starch hydrolyzed per gram of enzyme per hour (gS/gE-h) are compared in Table 3 for the three sequential fed-batch tests, Run 23 - 25. For Run 24, two situations are considered: first 3 cycles only and then all 4 cycles. Since the enzyme lost most of its activity after about 100 h on stream, only data up to about 100 hours of operation are reported in Table 3. The data from the first 150 h of the best continuous test, Run 22, is also included for comparison.

Table 3: Improvement in Enzyme Productivity.

	Run 22	Run 23	Run 24		Run 25
Process time, h	150	108	72.5	113	114
Numbers of cycles	-	3	3	4	3
Starch hydrolyzed, g	585	750	770	950	810
Enzyme usage, g	90	90	90	90	45
Hourly productivity*	0.043	0.077	0.12	0.09	0.16
Comment			reduce filtration time		half enzyme

* in grams starch hydrolyzed per gram enzyme per hour.

In Run 23, a total of 750 g of starch was hydrolyzed in 108 hours (first 3 cycles) using a total of 90 g of barley enzyme; therefore, the enzyme hourly productivity was about 0.077 gS/gE-h. In Run 24, the number of UF membranes were increased from 2 to 4 so the filtration time was cut by more than 2/3 in comparison to Run 23. For the same amount of enzyme, 950 g of starch was hydrolyzed in 113 h (4 cycles), and the enzyme productivity was increased to 0.093 gS/gE-h. If only the first 3 cycles in Run 24 are considered, this number becomes 0.116 gS/gE-h (although fewer cycles means more reactor down time). By reducing the enzyme usage to half in Run 25, 45 g of enzyme hydrolyzed 810 g starch in 114 h, the enzyme productivity was further increased to 0.16 gS/gE-h. Compared to Run 23, the enzyme productivity has been doubled. In comparison with Run 22, a four fold increase in the productivity has been achieved by optimization of the process operating conditions.

In conclusion, we have succeeded in optimizing the cold starch hydrolysis process using the membrane bioreactor at 40 °C and pH 4.5. In a sequential fed-batch operation using membranes for enzyme recycle, 15 g/L barley malt amylase has hydrolyzed three batches of starch of 110-130 g/L each over a total time period of 100 h. The hydrolysates contained an average of 100 g/L of fermentable sugar, ready to be fermented to ethanol.

4 STARCH HYDROLYSATE FERMENTATION

After starch has been converted by α-amylase through liquefaction and saccharification into fermentable sugar (hydrolysate), the next step was to study the yeast fermentation of sugar to ethanol. The reaction involved in the conversion of glucose to ethanol under anaerobic conditions can be summarized as:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

The maximum yield of ethanol from glucose is thus 0.511 g/g, although in practice yields are typically 90-95 % of this value.

We have selected a Muntons active brewing yeast for all our fermentation studies. The yeast inoculum was grown in a shake flask containing 100 ml of growth medium at 25 °C for 48 h prior to inoculation. The fermentation temperature was 30 °C and the fermentation medium pH about 4.2.

4.1. Batch Fermentation

We studied batch fermentation first using the starch hydrolysate from sequential fed-batch Run 25, batch 1. It had a total sugar concentration of 105 g/L as measured by the COD method. The hydrolysate was supplemented with yeast extract and minerals (KH_2PO_4, $MgSO_4$) for yeast growth. The fermentation was carried out anoxically in a 300 ml fermentor at 30 °C, initial pH 4.2 and 175 rpm agitation. Aerobic fermentation with an airflow of 1.2 L air/L broth/h was found to cause significant yeast growth but low ethanol yield. In addition, since many of the fermentable sugars in the hydrolysate were maltose and other oligosaccharides (determined by HPLC), a saccharification enzyme, glucoamylase (Sigma, catalog No A-7255), was added to the medium at 1 g/L at 30

minutes before yeast inoculation. Figure 15 shows how the yeast cell and ethanol concentrations increase during a batch fermentation test, Run 38. After a 5 h lag phase period, the cells proceeded to grow exponentially at a rate of about 0.9 g/L/h until they reached a stationary phase at 12 h with a maximum cell concentration of 6.5 g/L. The ethanol was produced rapidly during the exponential cell growth period, and the final ethanol concentration was 51 g/L. This gave a glucose to ethanol yield of 0.48 g/g, or 94 % of the theoretical yield for ethanol fermentation. Although the maximum ethanol was found from the samples taken after 22 h on stream, it had probably reached the maximum much earlier.

4.2. Continuous fermentation

Run 43

After the batch tests, we studied continuous culture fermentation next. First, a sequential fed batch hydrolysis run (Run 43) was conducted following the procedures of Run 25. From the first batch, 2.2 liters of hydrolysate with a fermentable sugar concentration of 100 g/L was collected. This hydrolysate, after adding glucoamylase and concentrated sterile nutrients, was then used for continuous fermentation without further sterilization. At a dilution rate 0.1 h^{-1}, the continuous culture fermentation achieved steady state operation for 40 h. Feed contamination then developed so the run was stopped.

Run 44

To overcome the problem of broth contamination, we then repeated the continuous fermentation under more strict aseptic conditions. An air breaker was installed between the feed tank and the feeding pump, which prevented possible

contamination in the fermentor from growing back to the feed. The initial batch fermentation medium was the hydrolysate from Run 25, batch 2 containing a fermentable sugar of 110 g/L (based on COD analysis). About 300 ml of sterile medium was first added to the fermentor aseptically. Gluocoamylase was dissolved and passed through a 0.2 micron syringe filter, the filtrate was next added to the fermentor. When the temperature reached 30 °C, 10 ml yeast inocolum was injected to the fermentor. After 14 h batch fermentation, the ethanol concentration reached 52 g/L and the yeast entered the stationary growth phase. At this time, continuous feeding began. The feed was a synthetic, sterile glucose solution of 90 g/L. Figure 16 shows the concentration profiles of the yield, glucose and ethanol during a 65 h continuous fermentation. In Figure 16, we saw drops in cell and ethanol concentrations soon after switching from batch fermentation to continuous culture, this was partly due to the lower glucose concentration (90 g/L) in the continuous feed than that in the initial batch medium (110 g/L). During continuous culture at a dilution rate of 0.1 h^{-1} and feed glucose 90 g/L, the steady state cell concentration was 3 g/L, residual glucose 6-7 g/L and ethanol 41-42 g/L. Therefore, the glucose conversion during the continuous fermentation was 93 %, and the cell yield was 0.036 g/g. The ethanol yield was again high, 0.49 g/g. The ethanol productivity was 4.1 g/L/h, which is typical for such continuous fermentation.

We are satisfied with these fermentation results. We have demonstrated that the hydrolysates from the cold starch hydrolysis can be efficiently converted to ethanol under our fermentation conditions. We also found that more stringent procedures need to be followed for collecting hydrolysates (UF permeates) and for formulating the fermentation broth, so that contamination can be avoided during continuous fermentation.

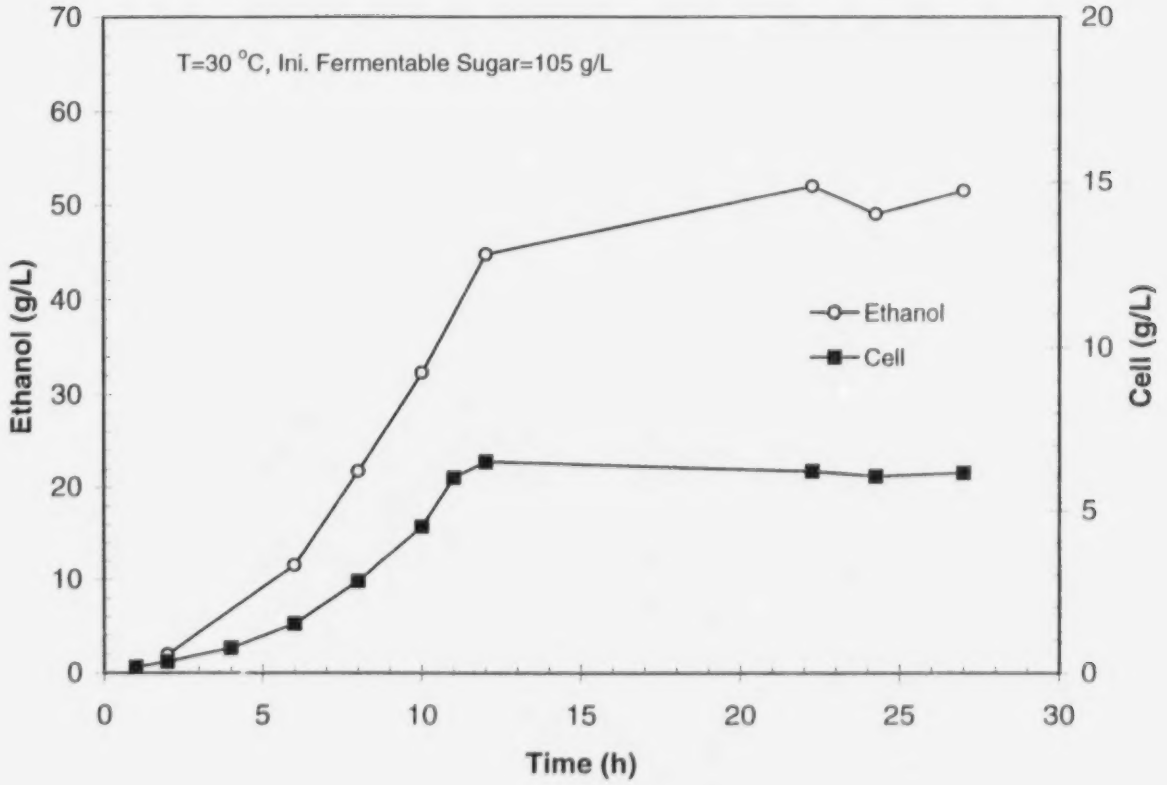


Figure 15. Yeast Cell and Ethanol Concentration Increase during Batch Fermentation Using Starch Hydrolysate (Run 38).

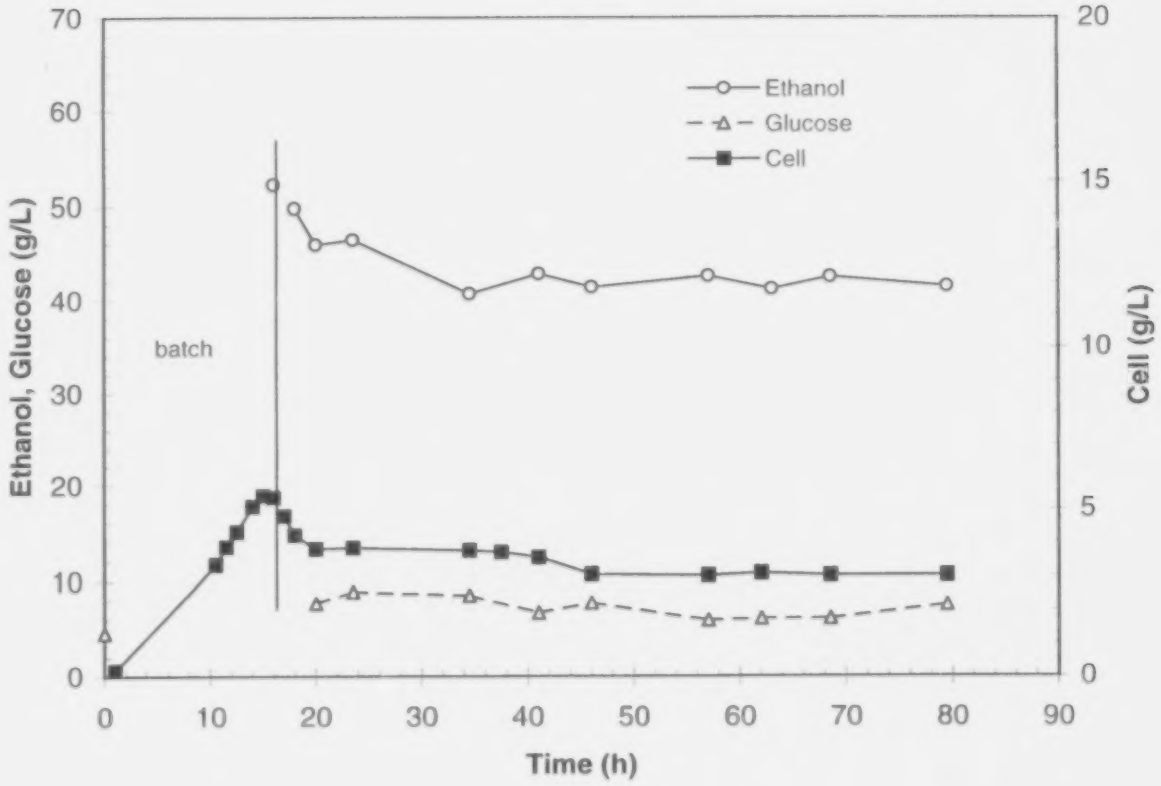


Figure 16. Concentration Profiles of Ethanol, Glucose and Cell During Continuous fermentation at 30 °C. Feed Glucose: 90 g/L, Dilution Rate: 0.1/h.

5. HYDROLYSIS OF STONE GROUND WHEAT

Stone ground wheat is more difficult to handle (hydrolyze) than pure starch. Besides starch, the ground whole wheat sample from Robin Hood Mills contains proteins and insoluble bran. The ground wheat particles are also much coarser; the mean diameter of the particles is about 300 microns, compared to only 20 microns for starch.

A few modifications were made in the bioreactor and in the analytic procedures to deal with these difficulties. First, to prevent large bran particles from entering into the membrane filters and thus clogging them, a 100 mesh stainless steel screen was welded over the end of the slurry withdrawal line inside the bioreactor to act as a prefilter. Second, because of interference from bran particles and the colour of the slurry, the spectroscopic method we used to determine starch granule concentrations was no longer suitable for analyzing ground wheat. We have tried a wet weight loss measurement in which 10 ml of slurry sample was taken from the bioreactor at different times of the reaction and filtered under vacuum through a 0.8 micron filter for 20 -30 minutes and the wet cake weighed. Each weight, w_t, is compared to the weight of the wet cake sample taken at the beginning of the reaction,, w_{t0}, and the difference was the wet weight loss (w_{t0}- w_t). We found the relative wet weight loss, (w_{t0} - w_t)/w_{t0}, to be comparable to the relative dry weight loss of the samples after they were dried (overnight) in the vacuum oven at 65 °C. The relative dry weight loss is directly related to the degree of starch hydrolysis, but it requires overnight determination. Therefore, we used the wet weight loss method for fast estimation of the starch conversions during ground wheat hydrolysis tests.

Run 31

The first hydrolysis of ground wheat was carried out at 40 °C, pH 4.5 and 15 g/L barley amylase loading (Run 31) with 0.004 M Ca^{2+} ions in the medium, and was planned as a sequential fed batch run. The wheat loading for the first batch was 186 g/L, corresponding to a starch loading about 120 g/L. After 3 h of batch hydrolysis, the wet weight loss was 32 %. It then slowly increased to 37 % at 6 h and to 40 % at 10 h. At 24 h, the wet weight loss reached 50 %, and the glucose concentration was 36 g/L. Although the conversion was lower than using pure starch, the reaction was stopped and the slurry was separated by cross flow membrane filtration. Because of insoluble fibers as well as lipids in the slurry, the filtration through the first stage microfiltration membrane was very slow and it took 12 h to complete (in comparison, 4 h was enough to filter a starch hydrolysate slurry). At the end of the separation, we discovered that the pH of the MF permeate was only 3.5 and no enzyme had survived this acidity; as a result, the next cycle of hydrolysis was abandoned.

We have investigated the problem of slurry acidity during whole wheat hydrolysis. The change in pH was monitored using a pH meter in wheat slurries both with and without adding enzyme, and in a third slurry where the wheat was pre-dried at 70 °C for 1 h. In all three situations, the slurry pH dropped to 3.5 after 24 h at 40 °C (Figure 17). Since we never experienced such problems when dealing with starch, and the operating procedures for wheat flour hydrolysis followed the same ones used for starch, we did not believe that the contamination stemmed from errant operations. Later, the phenomenon of changing pH was confirmed as the result of contamination by a certain yeast (Section 6.3). In the meantime, since barley malt amylase lost its activity

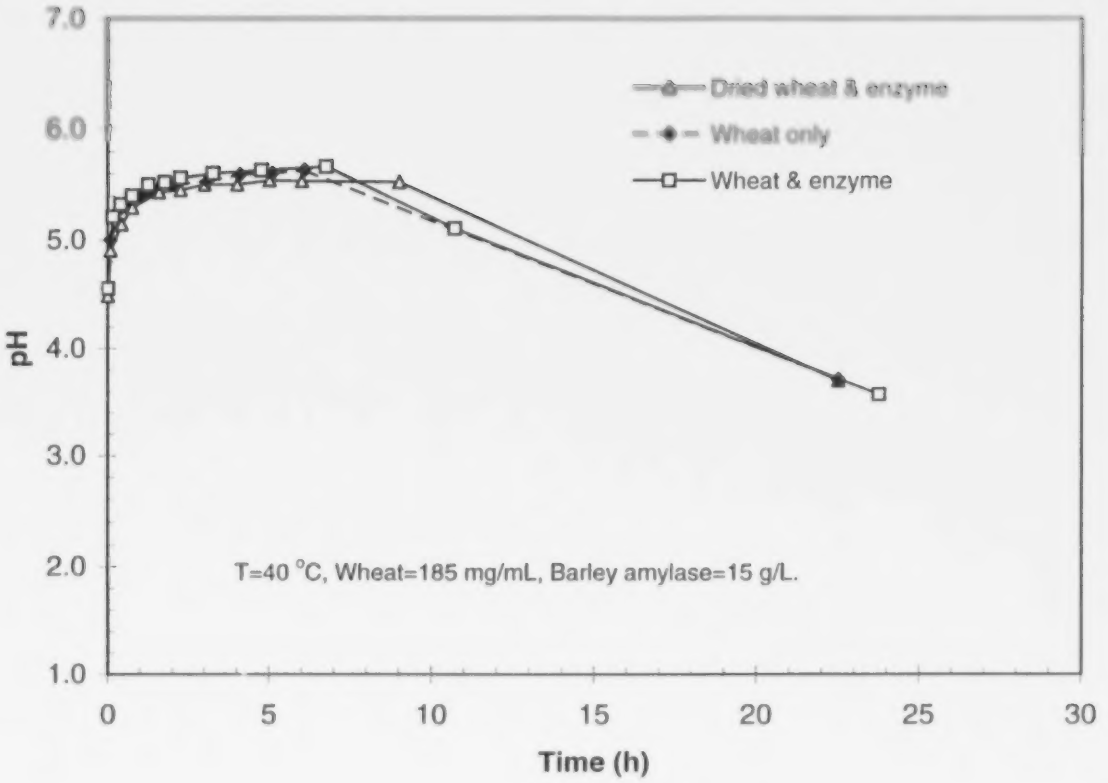


Figure 17. Variation of pH with Time in Wheat Slurries at 40 °C.

rapidly at pH < 4.0, we concluded that pH control was necessary during wheat hydrolysis.

Run 33: pH Controlled Hydrolysis

In Run 33, we installed a pH controller in the bioreactor that regulated the flow of a 7.5 N NaOH base solution to maintain the wheat slurry pH above 4.5, in order to protect the enzyme activity. The test conditions of Run 33 were the same as that of Run 31. After mixing the wheat slurry, its pH first went up from pH 4.5 (medium pH) to about pH 5.6. About 10 h later, the pH started to decrease. Over the next 12 h period, approximately 70 ml of the NaOH solution was added to the 3 L of wheat slurry in order to keep the pH at 4.5. This represented a base consumption of 0.5 mole of OH^- per liter slurry. As a result of maintaining the pH above 4.5, the remaining enzyme activity after 24 h hydrolysis was improved to 40 %. This demonstrates that accurate pH control is essential during the whole wheat hydrolysis process. Filtration was again very slow. Some bran particles leaked through the screen pre-filter and clogged the MF membrane downstream of the bioreactor, which led to the termination of the run.

Coarse wheat bran complicated both the hydrolysis and membrane filtration processes. It was then proposed that the cereal grain for ethanol production could be preprocessed to remove the bran fiber and increase the starch concentration in the feed-stock. Higher concentrations of starch in the feed would increase plant production and reduce energy consumption (Solulski, et al., 1997). We have decided, therefore, that the bran would be removed from ground wheat in future tests.

6. WHEAT FLOUR HYDROLYSIS AND FERMENTATION

Because a preprocessed wheat is preferred for real wheat hydrolysis, we have used an "untreated" flour as feed stock of cold hydrolysis studies. The main difference between this flour and previous stone ground whole wheat flour was that bran (fiber) had been removed by a simple sieving procedure following the milling. Again, due to the colour of the flour slurry, the spectroscopic method was unsuitable for starch granule measurement. Rather, the hydrolysis progress was monitored using the COD method, which measured the total organic compounds dissolved in the medium.

6.1. Flour Hydrolysis

Run 34

The "untreated" flour was tested in a sequential fed-batch hydrolysis test, Run 34. For the first batch, 130 g/L flour and 15 g/L barley malt amylase was charged to the bioreactor. After 22 h hydrolysis at 40 °C, pH 4.5 and 11 h membrane filtration, 2.0 liters hydrolysate (UF permeate) were collected. The COD of the hydrolysate was 92 g/L whereas the glucose concentration was 35 g/L. The second batch started with 125 g/L flour. After 38 h hydrolysis followed by 7 h filtration, 1.5 liters of hydrolysate was produced with a COD of 93 g/L, but a glucose concentration of only 27 g/L. The third and fourth batches lasted much longer, 110 and 90 h, respectively, and fresh enzymes were supplemented to the bioreactor during each batch hydrolysis. In comparison with whole wheat, the membrane filtration flux was higher when handling the bran-free flour slurry, and line clogging was not encountered. While the hydrolysates from batches 3 and 4 still had a total COD about 100 g/L, the glucose concentration decreased with time:

at the end of the fourth batch, the glucose in the hydrolysate was reduced to merely 3 g/L. The slurry pH was again controlled during the flour hydrolysis. Like in whole wheat hydrolysis, the strong 7.5 N NaOH base solution was employed to "neutralize" the continually formed acids in order to maintain the slurry pH at 4.5. Evolution of acid was suspected due to growth of a certain microorganism (contaminants) whose metabolism took up glucose to produce acid. This explains why the glucose concentration decreased during flour hydrolysis but the total COD remained high in the hydrolysates.

6.2. Flour Hydrolysate Fermentation

Run 39

The flour hydrolysate from Run 34, batch 3 (200 h on stream), having a COD 100 g/L but glucose 27 g/L, was later used in batch fermentation, Run 39. The fermentation conditions were the same as in Run 38 using starch hydrolysates as the carbon source: anoxic, 30 °C, pH 4.2, agitation and adding 1 g/L glucoamylase prior to yeast inoculation. The cell growth curve during the fermentation is presented in Figure 18. This figure shows that the exponential cell growth was again completed after 12 h with a maximum total cell concentration 2.7 g/L. The residual glucose after 24 h was 1 g/L, but the final ethanol was only 18 g/L. Since the total COD in the feed hydrolysate was 100 g/L, the fermentation yield was only 0.18 g/g, or merely 35 % of the theoretical yield. The composition of this hydrolysate was later analyzed by HPLC, and the total fermentable sugar was found to be only 41 g/L. If based on HPLC data rather than COD data for total fermentable sugar, the ethanol yield was instead 0.45 g/g, or 88 % of the theoretical yield. Therefore, the fermentation yield alone was acceptable. However,

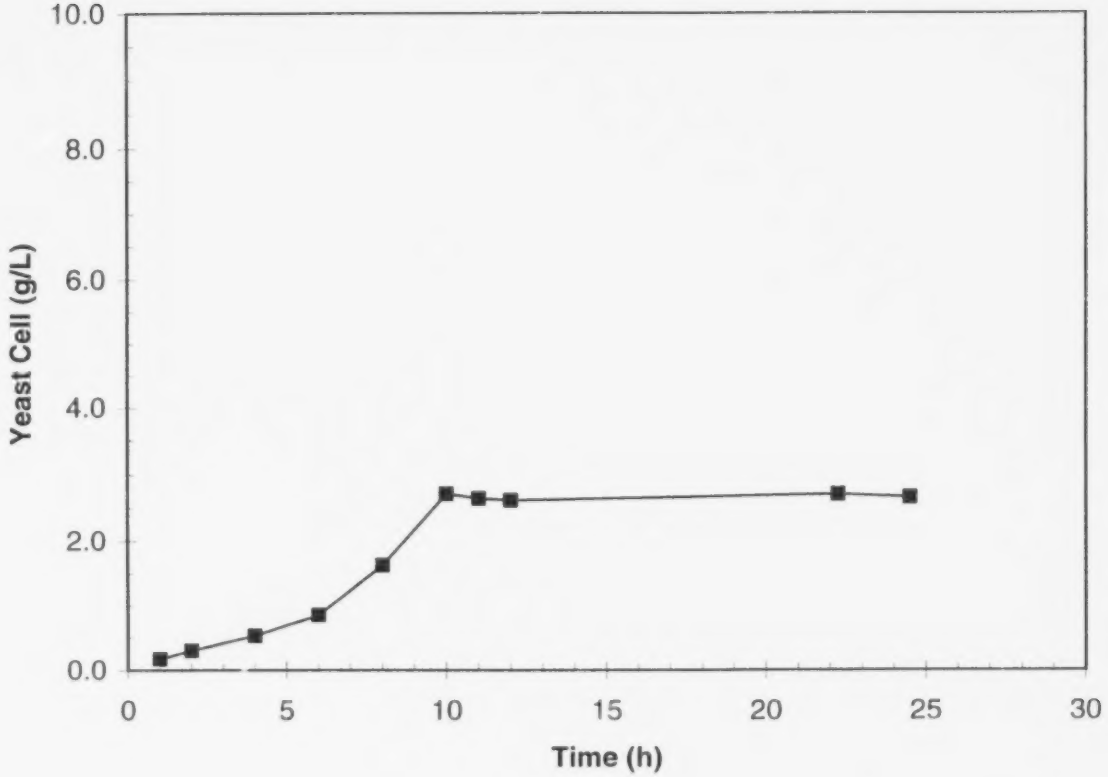


Figure 18. Cell Growth Curve from Fermentation Using Hydrolysate of Unsterilized Wheat Flour at 30 °C (Run 39).

considering about 100 g/L of COD producing 18 g/L of ethanol, the overall yield from flour starch to ethanol was unacceptable.

While the HPLC method quantitatively analyzed glucose to G7 fermentable sugars, the COD method determined not just the sugar but all oxidizable organics in the hydrolysate. Low ethanol concentrations and large discrepancies between the HPLC and COD values indicate that more than half the fermentable sugars derived from starch hydrolysis had been wasted in the bioreactor by some contaminating microorganism, probably in forming organic acids.

The microorganism contamination was not unique to this "Robin Hood" brand wheat flour; we found it also occurred in other commercial flours.

Runs 41 and 42: Using Sterile Flour

To overcome microorganism contamination, the "Robin Hood" flour was sterilized in the bioreactor by autoclaving at 121 °C for 60 min before hydrolysis. In Run 41 at 40 °C, the initial slurry pH was 4.8 by mixing 15 g/L of barley amylase and 130 g/L of flour with a medium of pH 4.5. The slurry pH did not change during the first 7 h hydrolysis, then started decreasing. However, 40 ml of the NaOH solution was consumed overnight for controlling the slurry pH at 4.5, showing that autoclaving did not fully destroy the contaminating microorganism and it began to grow again. The batch hydrolysis was stopped at 20 h, and the hydrolysate was collected through membrane filtration.

The hydrolysate of the sterile flour from Run 41 was used for fermentation in Run 42, under the same conditions as in Run 39. After 10 h, the maximum total cell concentration was 4.4 g/L and ethanol was 35 g/L. This ethanol concentration has

doubled compared to the 18 g/L of ethanol obtained in Run 39, but is still a low overall yield.

6.3. Identifying Contaminant

The slurry sample of the sterilized flour from Run 41 was also streaked onto agar plates. In 48 h, microbes were fully grown on the plates. The microbe has been identified by Norwest Lab (Edmonton, AB) as a single yeast species, *Candida fomata*. *C. fomata* is a common yeast, and could have adhered to the wheat during growth and later carried to the flour products.

To summarize our work with bran-free wheat flour, we have investigated both hydrolysis in sequential fed batch mode and batch fermentation. Removing insoluble particles from the whole wheat flour facilitated membrane filtration by both increasing filtration rate and avoiding membrane clogging. However, contamination by *Candida fomata* converted valuable fermentable sugars to unfermentable acids. This contamination severely compromised the overall efficiency of the hydrolysis-fermentation process if using untreated flour as the feedstock. Using sterile flour has improved the overall ethanol yield over a short period (in the first 20 h), but it did not completely eliminate the problem. Contamination would be a major dilemma over prolonged operations.

7 SIMULTANEOUS HYDROLYSIS AND FERMENTATION (SHF)

In the work reported so far, hydrolysis and fermentation have been studied separately. In the last part of our experimental studies, we attempted to combine these two steps into a single stage, starch to ethanol operation. In this simultaneous hydrolysis fermentation process, both biochemical reactions, cold hydrolysis (liquefaction and saccharification) of starch granules to glucose by enzymes and the subsequent fermentation to ethanol by brewing yeast took place at the same time in a single vessel:

$$\text{Hydrolysis: } (C_6H_{10}O_5)_n + n\ H_2O \rightarrow n\ C_6H_{12}O_6$$

$$\text{Fermentation: } C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

There are many advantages of using this method. First, because the glucose is utilized by yeast as it is formed, its inhibition on amylase activity would be abated. Near quantitative yields of glucose are possible, this will minimize possible reversion reactions catalyzed by glucoamylase at equilibrium glucose level. Furthermore, by preventing the accumulation of glucose, the batch SHF systems also minimizes bacterial contamination growth (Lewis, 1996). On the economic side, completing all reactions in a single vessel saves excessive use of surge tanks, pumps and flow controllers.

The progress made first in batch mode then in sequential fed-batch operation are described below. All the tests were done in the 3 L membrane bioreactor.

7.1. Batch Simultaneous Hydrolysis and Fermentation

The simultaneous process was studied in the 3 L membrane bioreactor. A modified fermentation medium formulation with the inclusion of sodium and calcium

ions was used for both enzyme reactions and yeast growth. The yeast inoculum was cultured in a shake flask for 48 h prior to inoculation.

Run 45: Starch as Feedstock

The first SHF batch study used wheat starch as substrate. To start, 130 g/L starch, 15 g/L barley malt amylase, 1 g/L glucoamylase and 40 ml/L yeast inoculum were mixed with the medium (pH 4.5) and pumped into the bioreactor. Since the normal hydrolysis temperature of 40 °C was too high for yeast fermentation, whereas the optimum fermentation temperature of 30 °C would greatly reduce enzyme activity, the initial reaction temperature was compromised to 35 °C. The temperature was then lowered to 30 °C after 6 h to facilitate yeast growth. Also, because both starch and yeast cells were measured at the same 600 nm wavelength by the spectrophotometer, their individual concentrations could not be analyzed by the OD method as they co-exist in the bioreactor. Only glucose and ethanol concentrations were monitored with time. The glucose results, measured by the glucose test strip, seemed too high to be correct, as the complexity of the broth interfered with the accuracy of the glucose strips. The trend of glucose, however, was that its concentration rapidly shot to a maximum within 4 h, then quickly decreased with time as ethanol was produced. The ethanol profile is shown in Figure 19, Curve (a). This curve shows ethanol had already been produced within 4 h (10 g/L) of commencement of the SHF reaction. The ethanol concentration increased rapidly to 55 g/L at 12 h (corresponding to the yeast exponential growth period) and then gradually increased to about 65 g/L after 24 h and further to 69 g/L at 51 h. About 95 % of the starch had been converted within the initial 24 h period. After 2 days, all starch had been converted. The residual glucose was about 4 g/L. Since 130 g/L starch can

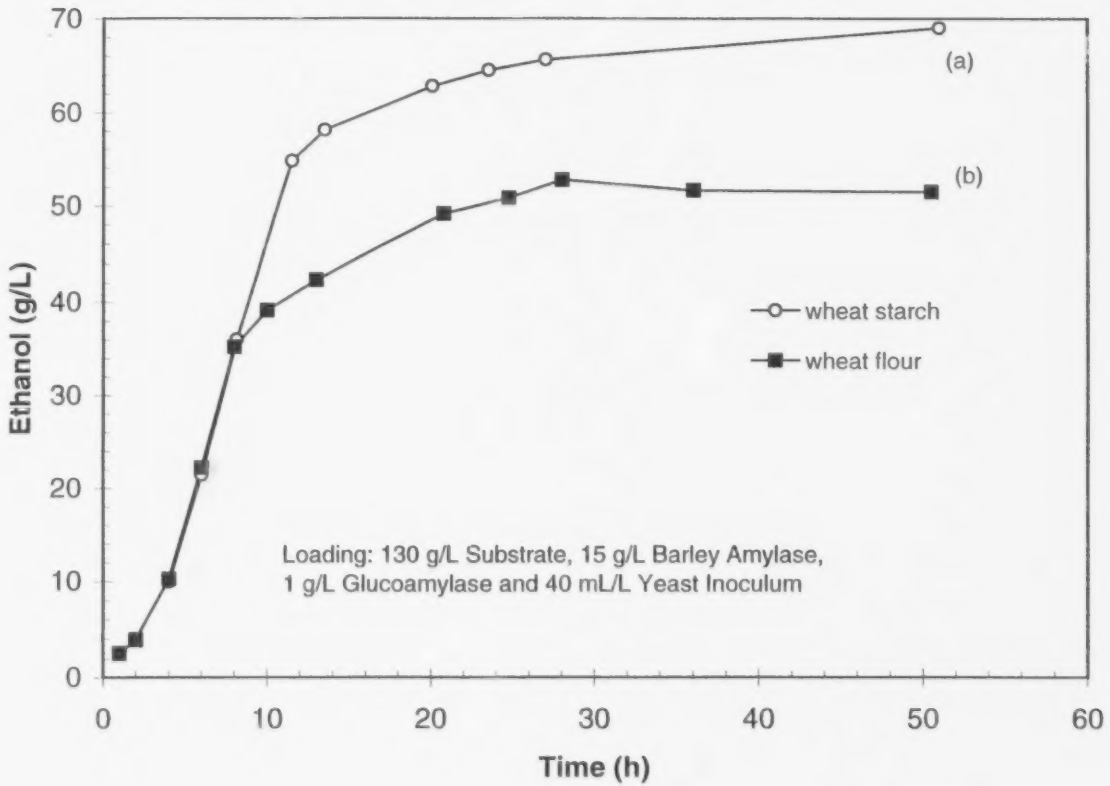


Figure 19. Ethanol Formation during Simultaneous Hydrolysis and Fermentation with (a): 130 g/L Starch (Run 45)m and (b): 130 g/L Flour (Run 46) as Substrates.

produce about 145 g/L glucose when fully hydrolyzed (1 g starch fully hydrolyzes to 1.11 g glucose), the ethanol yield based on glucose was 69/(145-4) = 0.49 g/g, or 96% of the theoretical yield.

The culture pH was 4.1 at 24 h on stream and was 4.2 at 51 h. Since the pH did not drop below 4.0, no contamination of *Candida fomata* occurred, even though the loading of starch, enzymes and yeast were performed in open air. This proves that *Candida fomata* originated from wheat flour itself rather than an airborne contamination in our laboratory.

The success of the SHF operation with starch was very encouraging: in a batch fermentor, about 95 % of a 13 wt% starch has been directly converted to ethanol with 96% of the theoretical yield. The product was a 6.5 % (w/v) ethanol solution, produced in 24 h at 30-35 °C.

Run 46: Flour as Feedstock

The "Robin Hood" flour, unsterilized, was tested next for simultaneous hydrolysis and fermentation in Run 46. Besides replacing starch with flour, the conditions and procedures of Runs 45 and 46 were the same. The ethanol profile when using a 130 g/L flour is shown in Figure 19, Curve (b). In this test, samples were taken at early stages for ethanol analyses. About 2.5 g/L ethanol had been detected at 1 h indicating that the yeast converted glucose soon after it was produced. This effectively alleviated glucose inhibition on amylase. The ethanol increased to 39 g/L at 10 h and after 24 h reached 51 g/L.

In comparison with the previous results when conducting hydrolysis and fermentation separately, we have made significant improvements both in overall ethanol

yield and in process simplicity. Previously, from a hydrolysate of the sterile flour of 130 g/L, 35 g/L ethanol was obtained after at least 2 days (Runs 41 and 42); if flour was not sterilized, its hydrolysate only produced 18 g/L ethanol (Runs 34 and 39). With SHF, 51 g/L ethanol was fermented from 130 g/L flour in 24 h.

There was another advantage of conducting SHF for the wheat flour feed stock. The culture pH in Run 46 was not controlled (i.e., no titration of NaOH); however, unlike in flour hydrolysis (alone), the pH remained at 4.2. Changing to pH 4.2 from an initial medium pH 4.5 was normal during ethanol fermentation because of carbonic acid formation. Therefore, by combining hydrolysis and fermentation reaction simultaneously, we have also overcome the problem of *C. fomata* contamination in flour hydrolysis. We speculate that due to a larger inoculum and favorable conditions, the brewing yeast outgrew *C. fomata* yeast in the reactor, suppressing its undesirable metabolism of converting sugar to acids.

In conclusion, we have demonstrated that both wheat starch and wheat flour granules can be converted to ethanol at high yields and at low temperature through simultaneous hydrolysis and fermentation in a batch fermentor. In addition, SHF has prevented the contamination of *C. fomata* when using flour feedstock and thus significantly improved the yield of ethanol production.

7.2. SHF in Sequential Fed Batch Operation

Following batch testing, we studied simultaneous hydrolysis and fermentation in a sequential fed-batch operation. In this way, both enzyme and yeast were recycled through membrane separation process and reused over several batches in order to reduce operating costs.

Run 48

Simultaneous hydrolysis fermentation was studied in Run 48 over a sequence of 5 fed batches. The basic conditions for each batch were similar to that of Run 45. The ethanol and glucose concentration profiles are shown in Figure 20 and the major event of the run is listed in Table 4. Following is the description of the run progress.

Batch 1 started with adding 130 g/L starch, 15 g/L barley amylase, 1 g/L glucoamylase and 40 ml/L yeast inocolum to the 3 L membrane bioreactor. The reaction temperature was 35 °C in the beginning; after 6 h it was reduced to 30 °C to facilitate fermentation. As shown in Figure 20, the glucose concentration increased to 90 g/L in 4 h, indicating that starch hydrolysis to glucose proceeded very quickly in this period. After that, as the yeast entered its exponential growth period, the rate of fermentation surpassed the rate of hydrolysis, so the glucose concentration started decreasing, whereas ethanol increased exponentially. The reaction was stopped at 24 h on stream and the membrane filtration process was started after 30 minutes of settling. The fermentation culture was found much easier to filter (for both MF and UF membranes) than the starch hydrolysate, since it was less viscous, containing smaller permeable molecules (ethanol vs. saccharides) and fewer starch granules. A total of 2.3 L of ethanol solution were collected in 4 h and the final ethanol concentration in the product, the UF permeate, was about 68 g/L. Therefore, the starch to ethanol yield was 0.52 g/g (=68/130). If all 130 g/L starch were converted to ethanol at the maximum yield, the ethanol would be about 73 g/L, (130*1.1*0.51=73, where 1.11 and 0.51 are the theoretical yields for starch to glucose and glucose to ethanol, respectively.). Therefore, the maximum starch to ethanol yield is 0.56 g/g (=73/130); and our ethanol yield in this batch was about 93 % of the

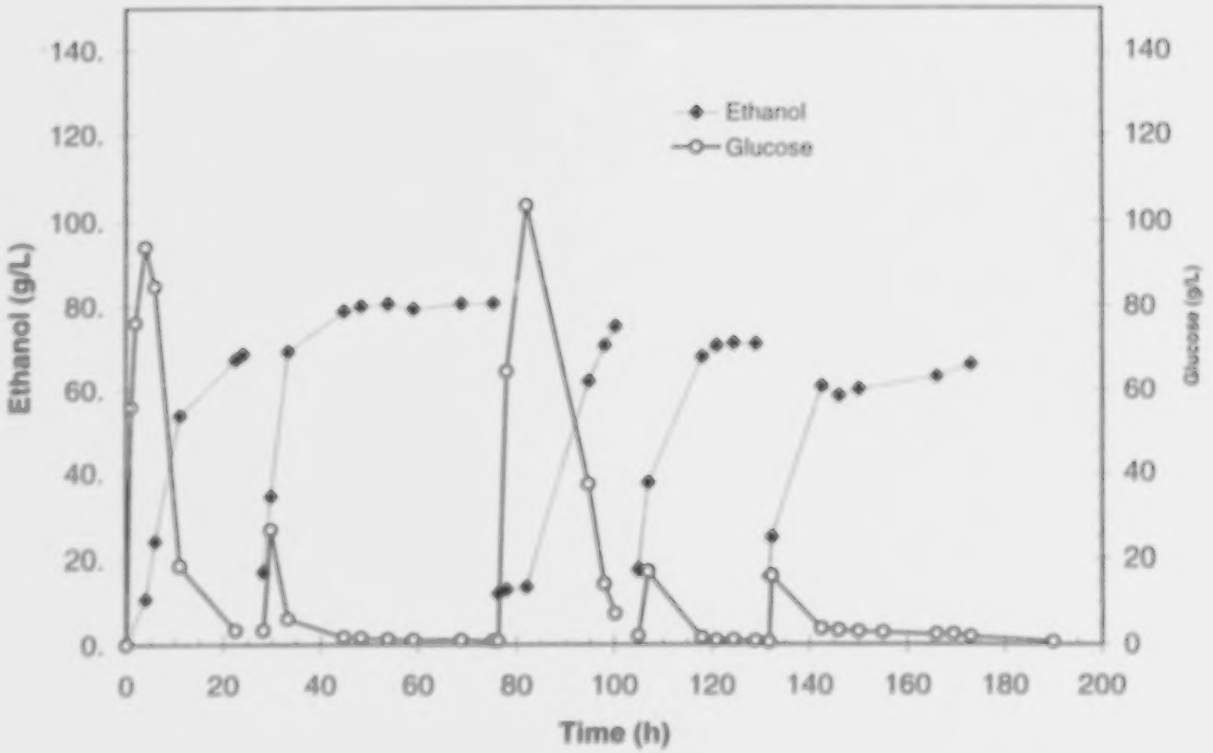


Figure 20. Simultaneous Hydrolysis and Fermentation in a Sequential Fed-Batch Process (Run 48).

Table 4. Major Events in Run 48:

Time, h	Event
0	Batch 1, load 130 g/L starch, 15 g/L barley amylase, 1 g/L glucoamylase, 40 ml/L yeast inocolum.
24-28	filtration, collect 2.3 L solution of 68 g/L ethanol.
28.3	Batch 2, load 120 g/L starch.
75.3	withdraw 2.5 L culture (no filtration) of 80 g/L ethanol.
76.3	Batch 3, load 130 g/L starch, 15 g/L barley amylase, 1 g/L glucoamylase.
100.3-104.5	filtration, collect 2.3 L solution of 75 g/L ethanol.
105	Batch 4, load 110 g/L starch.
129-131.5	filtration, collect 2.2 L solution of 72 g/L ethanol.
131.8	Batch 5, load 100 g/L starch
155	60 g/L ethanol concentration in the culture.
190	stop the run.

theoretical yield. At the end of this batch, about 0.7 L culture was left in the bioreactor containing all the enzymes and yeast cells.

Batch 2 started at 28 h after adding fresh starch slurry to the bioreactor. The total initial starch was about 123 g/L, whereas the initial ethanol was about 16 g/L (diluting 0.7 L of 68 g/L ethanol to 3 L). The yeast concentration was very high since all the yeast production during batch 1 was retained in the membrane bioreactor. Because of this,

glucose produced by hydrolysis was probably quickly consumed by the yeast to produce ethanol. As a result, there was no concentrated glucose build up in batch 2 as in batch 1; but instead, a rapid increases of ethanol without a lag phase was observed (Figure 20). The fermentation basically was completed at 48 h on stream (after 20 h batch time) with the ethanol concentration reaching 80 g/L. Because the initial ethanol was 16 g/L in the bioreactor, about 64 g/L ethanol was actually fermented during this batch from the feedstock of 123 g/L starch. This again was 93 % of the theoretical yield. The run was allowed to continue until 75 h. From 44 to 75 h, there was little change in ethanol concentration; on the other hand, glucose was continuously consumed for maintaining cell growth. The residual glucose was merely 0.8 g/l at 75 h. After 75 h, 2.5 L of culture with an ethanol concentration at 80 g/l was simply withdrawn from the bioreactor. The remaining 0.5 L culture served as the yeast inoculum for the next batch.

At 76 h, fresh starch and enzymes in fresh medium were pumped to the bioreactor to start batch 3. The initial starch concentration was 130 g/L, whereas the barley amylase was 15 g/L and glucoamylase 1 g/L, respectively. The left over ethanol was 13 g/L. In Figure 20, we observe a large glucose accumulation during the first 6 h, and at the same time a delay in ethanol formation. The yeast was unable to ferment glucose to ethanol promptly. It appears that yeast cells had lost viability and underwent a prolonged induction period after introducing the new medium. After that, the ethanol rapidly increased to 75 g/l at 100 h. The batch hydrolysis/fermentation was stopped after 24 h (at 100.3 h on stream) and the culture was filtered using membranes. The net starch to ethanol yield was 0.48 g/g (=(75-13)/130). The long induction caused a slightly lower yield in this batch.

Batch 4 started at 105 h by adding 110 g/L starch only and lasted for another 24 h. The behaviours of the glucose and ethanol curves were similar to that in batch 2, indicating high cell viability in the bioreactor. At the end (129 h on stream) the ethanol concentration reached 72 g/l. The filtration of batch 4 culture lasted only 2.5 h by optimizing the filtration conditions. It is significantly shorter than we ever managed for starch hydrolysate filtration.

The last batch, batch 5, started at 132 h by adding 100 g/L starch to the bioreactor. After 24 h or at 156 h on stream, ethanol was 62 g/L. The bioreactor was not shut down until 190 h, although there was little gain in ethanol concentration.

Less starch in each subsequent batch, from batch 3 to 5, was used. This was out of consideration that unreacted starch might build up inside the bioreactor with time. However, based on the final data available now, this was not necessary: the simultaneous hydrolysis and fermentation was very effective and residual starch from each batch was very small after a 24 h batch reaction

A few important conclusions can be drawn from Run 48:

(1) Except for the batch of yeast inoculation (batches 1 and 3), the glucose concentration was shown to be very low during the entire reaction time (batches 2, 4 and 5). Therefore, conducting simultaneous hydrolysis and fermentation in a sequential fed batch mode has abated the glucose inhibition on enzymes.

(2) Cell recycle enables high yeast concentration in the bioreactor, and thereby accelerates the ethanol production. The induction period in yeast ethanol fermentation was eliminated (or shortened) in batches 2, 4 and 5, and the exponential growth could be

completed in 10 to 12 h after fresh starch loading. Therefore, a batch period of 24 h should be enough for complete conversion of starch feedstock of about 130 g/L.

(3) Since enzyme inactivates significantly after 75 h, each fresh enzyme load can be reused for at least three sequential fed batches of SHF through membrane filtration.

(4) From a 130g/L starch feedstock, 63 g ethanol on average can be produced per liter of fermentation volume over a 24 h period. The starch to ethanol yield represents about 86 % of the theoretical yields.

(5) The filtration flux is about 37 L (broth)/m^2/h for the MF and 12 L/m^2/h for the UF membrane. Our filtration conditions are, however, not optimized.

There is no question that simultaneous "cold" hydrolysis and fermentation is more effective in converting starch to ethanol than performing the two processes separately. Therefore, the data of Run 48 will be used to perform the process economic evaluation for this project.

8. ECONOMIC EVALUATION

The plant design studied here is based on a wheat-ethanol process producing 10 million liter/year of 99.8 % ethanol. This size is identical to the Pound-Maker ethanol plant currently under operation at Lanigan, Saskatchewan. This design will use the new process of simultaneous cold starch hydrolysis and fermentation developed in this project.

8.1. Overall Process Description

By adopting the new process of simultaneous, cold starch hydrolysis and fermentation, the new plant will thereby eliminate the conventional, energy intensive, mash cooking step, and combine the liquefaction, saccharification and fermentation into a single operation. In order for enzyme and yeast recycle, a new membrane separation step is introduced to the process. By eliminating cooking, the new process not only saves energy, it also increases the sale value of its by-products, bran and feed protein, by protecting the nutrients from thermal degradation. A block diagram of the process is shown in Figure 21 and brief descriptions of the major process stages are given below. The numbers in the parentheses refer to the steps in the block diagram. More detailed unit operation conditions can be found in Warren et al. (1994); Lewis (1996) and in the Pound Maker (Lanigan) project final report (1988).

- *Feedstock preparation*: steps (1)-(5) and (12). Wheat is delivered by rail and cleaned and weighed. Grain is then crushed or milled, and sieved to separate the endosperm from the bran. The bran is stored and packaged as one of the by-products.

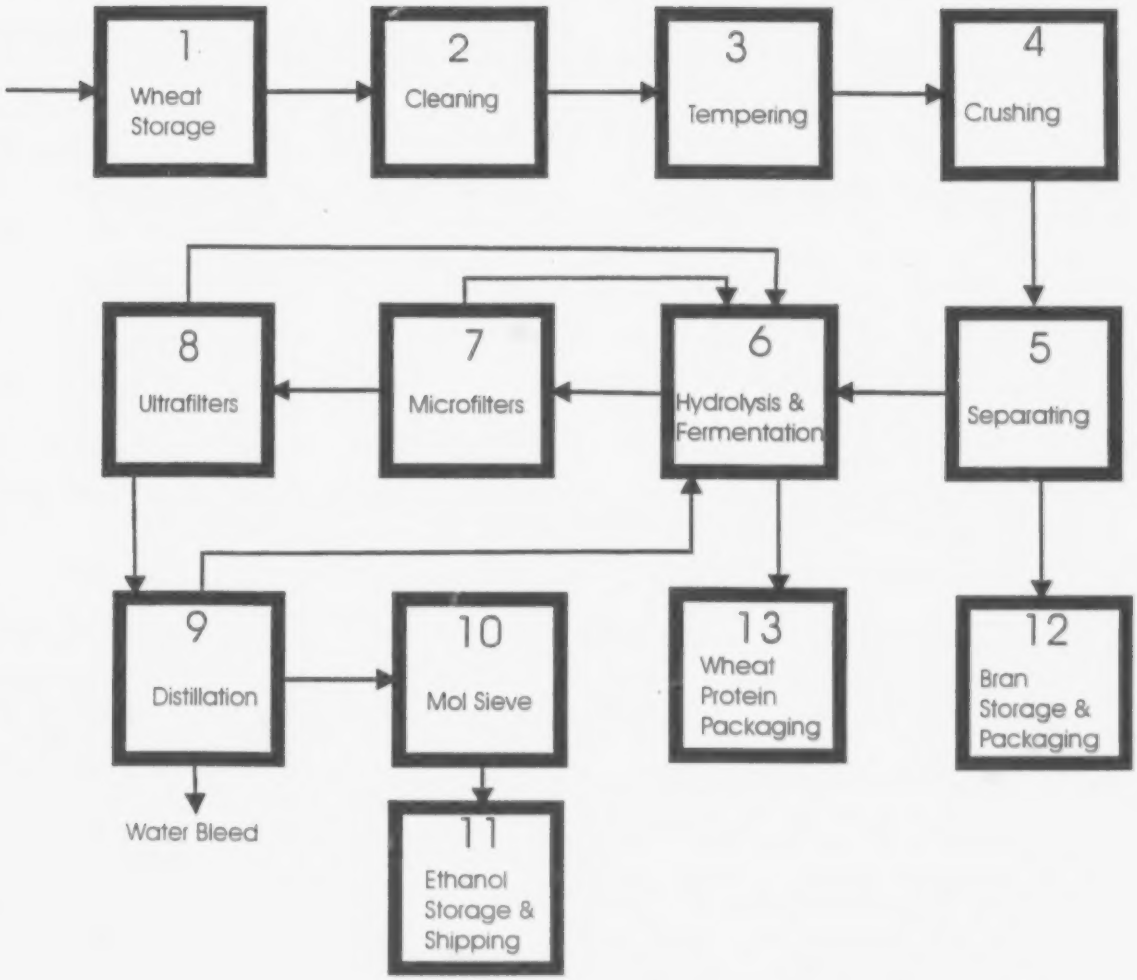


Figure 21. Block Diagram of the Designed Fermentation Ethanol Plant Process.

- *Simultaneous hydrolysis and fermentation*: step (6). The wheat flour is mixed with process water, barley malt amylase, glucoamylase and yeast, along with other necessary nutrients and chemicals in a sequential fed batch operation to directly produce ethanol at 30 – 35 °C.
- *Membrane filtration*: steps (7)-(8). At the end of the batch reaction, the broth is filtered through a two-stage microfiltration and ultrafiltraion process using hollow fiber membranes. The enzymes and yeast are recycled back to the fermentor, whereas the UF permeate is collected to a beer well. Each reaction/filtration cycle lasts 24 h. Each enzyme loading is used for three batches. The fermentor is then emptied and cleaned before recharging.
- *Ethanol concentration*: steps (9)-(11). The hydrolysate from the beer well is fed to a distillation column. The overhead product from the rectifying section is 94.5 % ethanol which is fed to the molecular sieve column to remove additional water. The 99.8% fuel grade ethanol is then condensed and collected in bonded storage tanks.
- *By-product recovery*: step (13). After every three batches, the retentate in the fermentor is discharged and the concentrate is prepared for sale as food protein.

8.2. Process Yields

The yields of revenue generating products, ethanol, wheat bran and proteins, from each tonne of wheat fed to the plant are the critical assumptions of this economic evaluation. We have consulted Robin Hood Mills of Saskatoon for information on the debranning process. For every 1,000 kg wheat, 150 kg bran can be separated by available

technology (Satake USA INC., Houston, TX). A theoretical calculation was made to determine the ethanol yield. The calculation was based on a wheat starch content of 65 wt% on a wet basis (10 % moisture). It was assumed that the hydrolysis yield is 98 %, fermentation yield 93 %; and the efficiency of the overall process is 95 %. The yield of CO_2 was acquired from the Lanigan report (1988). From a simple mass balance, the yield for the remaining wheat protein was estimated. The results are listed in Table 5.

Table 5. Yield from Wheat Feed (per tonne assuming 10 % moisture)

Product	Yield
Ethanol, L	400
(Ethanol, kg)	(315)
Bran, kg	150
Wheat protein, kg	160
CO_2	344

8.3. Evaluation of the SHF and Membrane Filtration Stages

The simultaneous hydrolysis fermentation and the coupling of membrane filtration for sequential fed batch operation are the major new unit operations in this plant design. Their economics are evaluated based on our new experimental data. In the following, we first determine the sizes of the major equipment, i.e., fermentor and membrane filters, as well as the quantities of enzymes. After that, we estimate the equipment and enzyme costs using unit prices from either vendor quotes or from the literature. All costs are in Canadian dollar unless specified otherwise.

Equipment Size and Enzyme Quantity

(1) *Size of fermentor*: Based on 330 operating days per year, the daily ethanol production of the plant is 30300 L (ethanol)/day. Using the data from Run 48, the daily ethanol production per fermentation volume is about 63 g (ethanol)/L/day, which is equivalent to 0.080 L (ethanol)/L/day. Therefore, the total fermentation volume required will be 380,000 liters.

Allowing for 25 % spare volume, the total fermentor volume will be about 500 m^3. The Lanigan plant currently has four 280 m^3 vessels as batch reactors for their production. We propose the use of three such 280 m^3 vessels in our design. Two vessels are used for fermentation at any one time (total volume of 560 m^3) with one vessel emptying, cleaning and filling.

(2) *Size of membranes*: The MF and UF membranes are designed to concentrate 200 m^3 fermentation broth by a factor of ten in 4 h, i.e., for a water removal rate of 45 m^3/h. A literature value of 35 $L/m^2/h$ (Tutunjian, 1985) is selected for the average MF and UF flux; therefore, the required membrane surface area is about 1,300 m^2 each for the MF and UF membranes, respectively. This large membrane area can be built by arranging smaller units in a rack.

(3) *Quantity of barley malt amylase*: Based on Run 48, 5 g barley amylase (15 g/L for three sequential batches) is needed for the production of 0.08 L ethanol. To make 10 million liters ethanol per year, the annual barley malt amylase consumption becomes about 625,000 kg/yr.

(4) *Quantity of glucoamylase*: The Lanigan plant currently adds 460 g Novo glucoamylase into the fermentor for each tonne of wheat, so the annual consumption is

about 12,000 kg/yr. The glucoamylase sample we used has been on the shelf over 2 years and its activity units were not measured. Since the functions of the saccharification enzyme should be the same in our process and in the conventional process used at Lanigan, we will use the current Lanigan value for our design.

Cost of Major Equipment and Enzymes

(1) *Fermentor cost*: The type of fermentor utilized for ethanol fermentation is a relatively simple vessel, made from either stainless steel or carbon steel. According to Maiorella et al. (1984), the unit price for a batch process fermentor was \$ 610/m^3. For a 280 m^3 vessel priced in 1998 Canadian dollars, each fermentor will cost \$210,000; or \$630,000 for total of three fermentors

(2) *Membrane cost*: We have received a quote from Millipore, Canada. The estimated cost for the hardware (including pumps) is \$ 1.5 million for each MF and UF unit, respectively. The cost for the membrane is \$1 million for MF and \$0.7 million for UF membranes, respectively. A 20 % discount off the quote is expected when ordering. The normal membrane life is 1 – 2 years. Since the membrane will be used only 8 h each day (4 h each for 2 reactors) during the sequential fed batch operation, we plan to replace the membranes every 4 years.

(3) *Unit cost of barley malt amylase*. Although no major enzyme manufacturers currently produce bulk quantities of barley malt amylase, one large US malting company, Malt Products Corp. (Indiana), offers barley malt flour with active enzymes at \$US 0.38/lb, or about \$Can 0.84/kg. We adopt this figure as an approximation for the bulk price of an industrial grade barley malt amylase.

(4) *Unit cost of glucoamylase:* The Novo glucoamylase used in Lanigan plant costs $4.4/kg. We will use this unit price for the calculation.

8.4. Total Capital Cost

Warren et al. (1994) have presented a cost evaluation for a 100 M liter/year wheat-ethanol plant and included the prices for various major equipment, besides the fermentor and membranes, based on the 1990 price index. We used those values and corrected with a time and size factors to estimate the equipment cost for a 10 million liter/year ethanol plant in 1998, according to the equation (Ulrich, 1984):

$$C_{(10,98)} = C_{(100,90)} \times \left(\frac{10}{100}\right)^{\alpha} \times \left(\frac{MSI98}{MSI90}\right) \qquad (3)$$

The size exponent, α, is 0.6, following the six-tenth rule for rapid estimation. The MSI is the Marshal & Swift Index, with MSI98 = 1060 and MSI90 = 915, respectively.

The purchase prices for the major equipment is summarized in Table 6.

Referring to the Lanigan project final report (1988) on land price, start up and other costs, the total required capital investment is calculated in Table 7. The total capital investment sums to about $8.33 million.

Table 6. Estimated Equipment Cost (in $Can 1,000)

Stage	Cost
Feed storage, Mill	290
Hydrolysis-Fermentation	630
Microfiltration	1,200
Ultrafiltration	1,200
Distillation	520
Evaporation	1,140
Product storage	200
By-product processing	380
Boiler	110
Total Equipment	5,670

Table 7. Total Capital Investment (in $Can 1,000).

Item	Cost	note
Feed	5,670	A
Contingency and fee	2,270	40 % of A
Total equipment	7,940	1.4 A
Land	0	As Lanigan
Startup cost	60	B
Fixed capital cost	8,000	1.4 A + B
Working capital	330	(1 mo raw material + labor)
Total Capital Investment	8,330	

8.5. Operating Cost

The plant annual operating cost includes the expenses on raw materials, replacement of membranes, as well as utilities, personal and maintenance, etc.

About 25,000 tonnes of wheat is needed for the 10 million liters of ethanol production each year. The wheat price in Saskatchewan can be estimated from the price listed in the Winnipeg commodity future market, subtracting $30-40 for freight savings. Using this method, the wheat price is projected at $120/tonne. The annual wheat feedstock cost reaches $3 million.

Based on the data in Section 8.3, the total enzyme cost is $580,000 each year. The allocated annual costs for membrane replacement are $200,000 for MF and $140,000 for UF, respectively.

The Lanigan plant employs a total of 14 personnel. Since our process is simpler by combining cooking, saccharification and fermentation into one operation, we propose a total of 12 employees for the new plant at an average annual cost of $30,000 each.

The utilities, maintenance and insurance costs are calculated based on the Lanigan report (1988) after adjusting for inflation. For utilities, an additional 15 % savings is adjusted because of the removal of the energy consuming mash-cooking step.

The annual operating cost of $5,240,000 or 52.4¢ per liter of ethanol has been calculated, and the breakdown expenditure is summarized in Table 8.

Table 8. Total Operating Cost (in $Can 1,000).

Item	Cost
Wheat	3,000
Enzymes	580
Other Chemicals	10
MF membrane	200
UF membrane	140
Utilities	450
Maintenance, insurance	500
Labor	360
Total Capital Investment	5,240

8.6. Revenue

Ethanol

The price of fermentation ethanol produced in Saskatchewan needs to be competitive with the ethanol price in the US, which in turn varies daily according to the fluctuation in US corn price. Over the last three years, the ethanol price in US has varied from $US 0.95/gal. (May 1998) to $US 1.61/gal. (Oct. 1996). When converted to the Canadian dollar, an average ethanol price of 46¢/L or $460/m^3 is considered to be reasonably projected. For an annual production of 10 million liters, the total revenue from ethanol sales is, thus, $4.6 million.

By-products

The by-products from the conventional whole grain to fermentation ethanol processes are the so-called DDG (Dried Distillers Grains) and/or DDGS (Dried Distillers Grains and Solubles), and are marketed for animal feed.

The by-products from this new design are different. Bran will be separated from the endosperm prior to the hydrolysis/fermentation stage, and collected as one of the by-products. From 25,000 tonnes of wheat per year, we will obtain 3,750 tonnes of bran, and when sold at a price of $100/tonne (Robin Hood Mill, private communication) for animal feed, the generated income is $375,000 per year.

The concentrated or dried retentate from the hydrolysis/fermentation process is processed as the other by–product. It is mainly made of proteins (wheat gluten), sugars and yeast cells, etc. Since it is not thermally denatured by cold hydrolysis, it will be marketed as a food protein for human consumption similar to wheat gluten. We used the bulk price of wheat gluten to approximate the value of this new product. Facing strong

competition from the European Union producers, the wheat gluten price in the USA fell 30 % in the last two years to US 47 ¢/lb (Seaberg, 1998), or $Can 1.50/kg. About 4,000 tonnes of such proteins will be produced per year. It will bring in an annual revenue of $6.0 million for the plant.

The total revenue from the sales of ethanol and by-products, therefore, amounts to 10.9 million.

8.7. Project Economics (cash flow)

Economic Assumptions

- Plant size – 10,000,000 liters per year fuel grade ethanol with 3,750 tonnes of bran and 4,000 tonnes of wheat protein, respectively, from 25,000 tonnes of wheat.
- Ethanol Sales Price – 46¢/L
- Bran Sales Price – $100/tonne
- Wheat Protein Sales Price – $1.50/kg
- Construction Time – one year
- Project Life – 20 years
- Depreciation – 10 years, at a rate of (10-n)/55 for the nth year
- Taxes – 35 % including both Federal and Provincial taxes
- Stream Days – 330 days per year

Project Returns

Table 9 gathers all the financial data together in an annualized cash flow statement for the duration of the project life. The capital cost and the start-up cost are

reflected in the initial year. The plant will operate at 80 % of the designed capacity during the first year following construction and in full capacity thereafter. The total project life is 20 years after construction. At the end of that time, 12.5 % of the original capital investment, $1.04 million, will be recovered as salvage. The first column in Table 9 lists the year number, the other headings represent:

- ATC – Annual Total Capital Investiment
- AS – Annual Sale
- ATE – Annual Total Expenses (operating costs)
- AD – Annual Depreciation
- AIT – Annual Income Tax
- ANCI – Annual Net Cash Income
- + ANCI – Cumulative Annual Net Cash Income
- I – Interest
- fd – Depreciation Factor
- ADCF – Annual Discounted Cash Flow
- + ADCF – Cumulative Annual Discounted Cash Flow

Table 9. Annualized Cash Flow Statement (in $Can 1,000).

Year	ATC	AS	ATE	AD	AIT	ANCI	+ANCI	i= fd	0.1 ADCF	+ADCF	i= fd	0.46 ADCF	+ADCF
1	8330	0	0	0	0	-8330	-8330	0.91	-7573	-7573	0.68	-5694	-5694
2	0	8780	4194	1336	1138	3449	-4881	0.83	2850	-4722	0.47	1611	-4082
3	0	10975	5242	1203	1586	4147	-734	0.75	3116	-1606	0.32	1324	-2758
4	0	10975	5242	1069	1632	4101	3367	0.68	2801	1194	0.22	895	-1863
5	0	10975	5242	935	1679	4054	7421	0.62	2517	3712	0.15	605	-1258
6	0	10975	5242	802	1726	4007	11428	0.56	2262	5973	0.10	409	-849
7	0	10975	5242	668	1773	3960	15388	0.51	2032	8006	0.07	276	-573
8	0	10975	5242	535	1819	3914	19302	0.47	1826	9831	0.05	186	-387
9	0	10975	5242	401	1866	3867	23168	0.42	1640	11471	0.03	126	-261
10	0	10975	5242	267	1913	3820	26988	0.39	1473	12944	0.02	85	-176
11	0	10975	5242	134	1960	3773	30762	0.35	1322	14267	0.02	57	-118
12	0	10975	5242	0	2007	3726	34488	0.32	1187	15454	0.01	39	-80
13	0	10975	5242	0	2007	3726	38215	0.29	1079	16533	0.01	26	-53
14	0	10975	5242	0	2007	3726	41941	0.26	981	17515	0.00	18	-35
15	0	10975	5242	0	2007	3726	45668	0.24	892	18407	0.00	12	-23
16	0	10975	5242	0	2007	3726	49394	0.22	811	19218	0.00	8	-14
17	0	10975	5242	0	2007	3726	53120	0.20	737	19955	0.00	6	-8
18	0	10975	5242	0	2007	3726	56847	0.18	670	20625	0.00	4	-4
19	0	10975	5242	0	2007	3726	60573	0.16	609	21234	0.00	3	-2
20	0	10975	5242	0	2007	3726	64300	0.15	554	21788	0.00	2	0
21	-1040	10975	5242	0	2007	4766	69066	0.14	644	22432	0.00	2	2

Three different scenarios of interest rate or capital return rate are presented in Table 9, and also plotted in Figure 22. They are: 0 %; 10 % interest rate on investment capital; and the Discounted Cash Flow rate of return. In the last three columns, the maximum capital return rate is calculated by setting the final cash flow value at zero at the end of the plant life. The maximum return rate for the potential investors is calculated to be 46 % using this new cold hydrolysis technology.

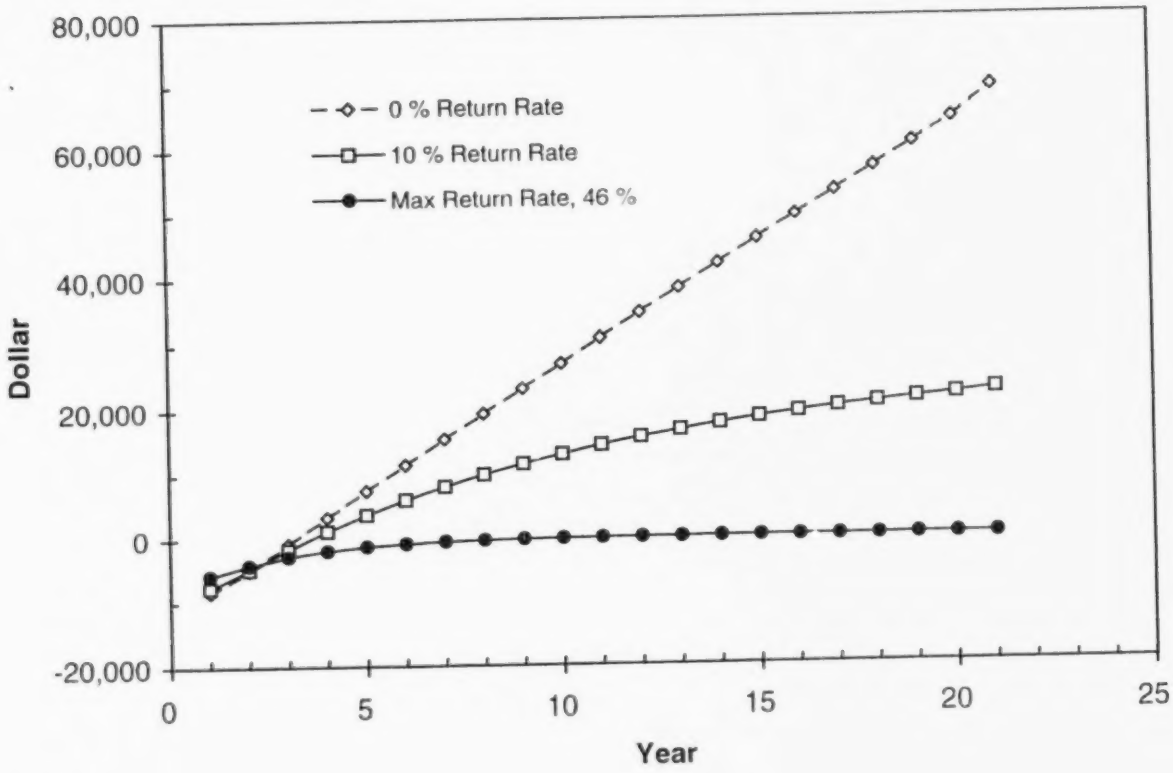


Figure 22. Investment Return at Different Interest Rate.

9. CONCLUSIONS AND RECOMMENDATIONS

We have developed a feasible hydrolysis process at temperatures below the starch gelatinization temperature for the manufacture of fermentation ethanol from Saskatchewan wheat. In this process, simultaneous application of barley malt amylase, glucoamylase and brewing yeast on debranned wheat flour combines the hydrolysis of wheat starch to sugar and the subsequent fermentation to ethanol into a one stage operation. As a result, ethanol can be produced with an overall starch to ethanol yield of at least 86 % in a single vessel, at 30 to 35 °C within 24 h. The soluble enzymes are reused through membrane filtration in a sequential fed batch manner and can last up to 75 h.

Our new process has much merit. It significantly decreases the production time of ethanol from wheat starch in comparison with the conventional multistage process. By eliminating the energy intensive, mash-cooking step, it is also more energy efficient. In addition, without thermal denaturing, our process produces a wheat protein by-product similar to wheat gluten that is suitable for human consumption.

The preliminary economic evaluation based on our new process for a commercial, 10 million liter per year ethanol plant shows a favorable Discounted Cash Flow Rate of Return of 46 % and a Payback Period of 27 months. We recommend, therefore, that this process is worthy of further investigation and some aspects should be advanced to Pilot Scale development.

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11. APPENDIX

1. Hill, G. A., D. G. Macdonald and X. Lang, "α-Amylase Inhibition and Inactivation During Cold Starch Hydrolysis", Biotechnol. Let. 19, 1139-1141, (1997).

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α-Amylase inhibition and inactivation in barley malt during cold starch hydrolysis

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α-Amylase from barley malt decayed at a rate of 1.1% h^{-1} at 45°C and this decay rate was greatly accelerated at 55°C. Glucose and maltose inhibited the catalytic actions of both bacterial and barley α-amylase in the same manner during the hydrolysis of starch granules. The catalytic activity dropped exponentially as these sugars increased up to 400 g/L; with about 50% activity at 100 g/L. Equations are presented to model both time decay of the enzyme and the inhibition effect of the sugars.

Introduction

Several industrial processes use α-amylase to hydrolyse starch prior to fermentation or production of added-valued biochemicals. This enzyme is known to effectively attack both soluble starch and starch granules held in an aqueous suspension. Before designing a successful hydrolysis system, information is required describing phenomena which affect the kinetics of starch hydrolysis by α-amylase. It is well-known that both proteins and hydrolysis products can inhibit the action of α-amylase on starch (Robyt, 1984), and so kinetic models must include these effects as well as enzyme decay if reliable predictions are to be made. Schwimmer (1950) indicated that end products inhibited hydrolysis kinetics in both a competitive and non-competitive fashion with a maximum specific hydrolysis rate for dissolved starch of 15 h^{-1}. Weselake and Hill (1983) found that maltose inhibited starch hydrolysis by a non-competitive mechanism but Houng *et al.* (1992) reported the inhibition by maltose was competitive. In order to account for enzyme decay with time, Nicholls *et al.* (1974) proposed an exponential time decay function and found that arylsulfatase had a decay constant of only a few minutes at 37°C. Houng *et al.* (1992) later applied a complex form of this exponential time decay function and reported that α-amylase was not completely deactivated until 48 h at 40°C. This agrees with the earlier work of Rollings and Thompson (1984a,b).

In this work, the decay and product inhibition effects for α-amylase hydrolyzing wheat starch granules at high concentrations are presented and mathematically modelled.

Material and methods

Enzymes and chemicals

Two enzymes were used in this work, both purchased from Sigma: bacterial and barley α-amylases. Wheat Starch 4 produced by Archer-Daniels-Midland (Montreal, Canada) was the substrate and consists of granules with a mean diameter of 21 μm (measured locally with a laser paticle size analyzer). The inhibiting chemicals, glucose and maltose, were of reagent grade and were puchased from VWR Scientific. The reactions were studied in an aqueous environment at pH 5.5 using distilled, deionized water with 0.002 M $CaCl_2$ (required to stabilize the enzyme) in very well-mixed, baffled vessels.

Methods and analyses

The aqueous, sterile environment inside the well-mixed reactors was first brought to temperature in an environmental chamber at which point the powdered enzyme and sugar were added and stirred for 15 minutes. Then the starch granules were added to begin the reactions which were sampled for up to 6 hours using sterile syringes. Samples were analyzed for starch granule concentration using a spectrophotometric method which had been calibrated and found to give reproducible readings within ± 5%. Enzyme activity was measured using an α-amylase activity kit supplied by Sigma. This kit provided reproducible measurements within ± 3%.

Results and discussion

Figure 1 shows data from three experiments measuring the deactivation of barley α-amylase, one experiment at 55°C and two experiments at 45°C. The scatter in data

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other sites on the α-amylase enzyme, since both competitive and non-competitive inhibition models failed to predict this behavior. It could be an osmotic effect caused by these very high sugar concentrations reducing the water activity in the vicinity of the enzyme-starch complex.

Both Equations 1 and 2 are now valuable additional information needed for long-term predictions of the behaviour of an industrial reactor. Equation 1 will help to identify the continuous decline in bioreactor performance due to enzyme decay and can be used to plan for new enzyme additions. Equation 2 shows that when the hydrolysis product concentration reaches 100 g/L (sugar concentrations needed for fermentation alcohol), the operation of a continuously well-mixed reactor would not be a favorable design strategy due to a drop of about 50% in enzyme activity. This suggests that an enzyme starch hydrolysis reactor might best be operated in either batch or fed-batch mode, or for continuous operation, a plug-flow tubular reactor should be considered.

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Cold Enzyme Hydrolysis of Wheat Starch Granules

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Three α-amylase enzymes were used to hydrolyze wheat starch granules suspended in water below the gelatinization temperature. The rates of hydrolysis were determined at various temperatures, pH, enzyme and starch concentrations. Barley amylase was found to be the "best" enzyme when used at pH 4.5, 45°C and starch and enzyme concentrations of 30 and 8 mg/mL respectively. It was found that under these conditions, 98% of the starch granules were hydrolyzed in 3 hours, the same amount of time used in the industrial cooking process of soluble starch. Starch particles were observed to be attacked at specific points on the surface and then hydrolyzed from the inside-out. Some granules were hydrolyzed at a very fast rate with a first order rate constant estimated to be 40 h^{-1}; but most granules were hydrolyzed slowly according to the Michaelis-Menten model and the best fit parameters were found to depend on enzyme type, pH and temperature.

On a utilisé trois enzymes α-amylase pour hydrolyser des granules d'amidon de blé suspendus dans l'eau sous la température de gélatinisation. Les vitesses d'hydrolyse ont été déterminées à des températures, pH et concentrations d'enzymes et d'amidon variés. On a trouvé que l'amylase de l'orge était le "meilleur" enzyme lorsqu'il est utilisé à un pH de 4,5, à 45° et à des concentrations d'amidon de 30 et 8 mg/mL, respectivement. On a trouvé que dans ces conditions, 98% des granules d'amidon étaient hydrolysés en 3 heures, soit le même temps utilisé dans le procédé de cuisson industriel de l'amidon soluble. On a remarqué que les particules d'amidon étaient attaquées en des points spécifiques de la surface puis qu'elles étaient hydrolysées de l'intérieur vers l'extérieur. Certains granules ont été hydrolysés à une vitesse très rapide avec une constante de vitesse de premier ordre estimée à 40 h^{-1}; toutefois, la pluspart des granules ont été hydrolysés lentement selon le modèle de Michaelis-Menten et on a trouvé que les meilleurs paramètres de calage dépendaient du type d'enzyme, du pH et de la température.

Keywords: enzyme hydrolysis, amylase, starch granules, modeling.

Approximately 25 million tonnes of Canadian wheat are grown each year, 50 % of which is produced in Saskatchewan. The starch content of this wheat is about 65% based on a dry mass basis. Starch has become a very important biopolymer and is used in many industries as a feedstock material (Bikales, 1970). In several industrial processes, enzymes are used to quickly transform starch molecules to useful, added-value biochemicals. Examples of two industries that have taken advantage of this development are the sweetener and ethanol industries.

Wheat starch is composed of approximately three quarters amylopectin and one quarter amylose, with small amounts of protein and lipid impurities (0.8% and 0.2% respectively, Bowler et al., 1985). Amylose is primarily a straight chain polymer with α-1,4 glycosidic bonds and only 4 to 5 branch points per molecule. Wheat amylose has a molar mass ranging from 100,000 to 300,000 Daltons (Bikales, 1970). Amylopectin is a branched polymer with α-1,4 glycosidic straight linkages and about 250 branch points per molecule. The structure of amylopectin is a grape-like cluster (Hizukuri, 1986; Robin et al., 1974) and it has a molar mass which ranges from 1 to 1.2 million. The amylose and amylopectin molecules are stored by plants in a granular form. Industrially, wheat starch granules are separated into two size ranges: 2–15 μm and 15–35 μm (Dronzek et al., 1972; Evers and McDermott, 1970). The small granules make up 95% of the total number of granules but the large granules compose 90% of the total weight.

Nigam and Singh (1995) have recently reviewed many of the papers dealing with the mode and kinetics of soluble starch hydrolysis. α-Amylase (α-1,4 glucan glucanohydrolase, EC 3211) attacks the internal α-1,4 glycosidic bonds of starch producing a rapid decrease in viscosity (Park and Rollings, 1994). The action pattern of α-amylase depends on the source of the enzyme but is known to be random on large molecules and less random as the molecules become smaller. In industry, α-amylase is used to convert starch into G5 to G7 molecules (liquefaction stage); which can then be converted to glucose by glucoamylase (saccharification stage; Reilly, 1985; Robyt, 1984). It is important to note that in industrial processes, these enzyme reactions are carried out at high temperatures (> 90°C) with the starch granules dissolved in water. Given similar concentrations of starch and enzyme, the hydrolysis kinetics of soluble starch is approximately an order of magnitude higher than starch granule hydrolysis (Morita and Fukuoka, 1997).

The distillation process used to purify ethanol from fermentation broth has been shown to be the largest single component of energy demand for a fermentation ethanol process (Warren et al, 1994). However, in 1987, Han et al. achieved a 17% energy savings in the overall production of ethanol by lowering the cooking temperature. Mikuni et al. (1987) report that energy savings could reach as much as 40% of the overall energy requirements by completely eliminating the cooking step. The removal of the cooking stage also has the potential to increase the value of the co-products since valuable proteins would undergo less thermal stress. The production of undenatured protein would be worth two to three times that of the denatured product (St. Denis, 1993). There could also be other benefits such as the extended use of enzymes due to diminished thermal denaturing and the possibility of a single-stage, starch to ethanol process.

In this study, α-amylase enzymes have been used to hydrolyze raw wheat starch granules at various concentrations, temperatures and pH using small, well-mixed batch reactors. A dual mechanism, kinetic model incorporating Michaelis-Menten kinetics was found to represent the experimental data extremely well.

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Model for Starch Granule Hydrolysis

Starch granules are insoluble in cold water. The kinetics of enzymes in solution acting on insoluble substrates has been reviewed by Bailey and Ollis (1986). The analysis requires consideration of the sites where the enzyme is attaching to the surface of the granule. In this situation, the reaction rate will reach a limit as the enzyme concentration is increased. To develop a model, the normal roles of substrate and enzyme are reversed and we assume the enzyme adsorbs onto the surface of the substrate (Bailey and Ollis, 1986; MacGregor and MacGregor, 1985; McLaren and Packer, 1970). The following equilibrium model is then proposed:

$$E + A \underset{kdes}{\overset{kads}{\rightleftarrows}} EA \xrightarrow{k_3} E + P \quad \ldots\ldots\ldots\ldots (1)$$

In Expression 1, E represents the free enzyme, A is an empty adsorption site on the granule, EA is the enzyme adsorbed on an adsorption site and P is the product of granule hydrolysis (water soluble). In this work it was observed that the kinetic disappearance of granules occurred by a dual reaction mechanism. If A_0 is the total number of original adsorption sites; A_{01} represents the sites where very fast hydrolysis occurs and A_{02} represents sites which are slowly hydrolyzed. Macregor and Ballance (1980) also observed this dual rate hydrolysis phenomenon and speculated that it was due to the ease of hydrolysis of mechanically damaged granules compared to undamaged granules. Another hypothesis has been that the rate of hydrolysis could be related to the starch molecule orientation, chain length or degree of branching (Franco et al., 1997; Manelius et al., 1997). The conversion of A_{01} to P occurs within the first few minutes after mixing the enzyme with the starch granules such that this can be modeled by a fast, first order reaction:

$$A_{01} \xrightarrow{k_1} P \quad \ldots\ldots\ldots\ldots (2)$$

Since the enzyme was in great excess in all cases of this study and measurements were made after six minutes from the time of mixing the reactants, Reaction 2 was found to be independent of enzyme concentration as well as all other operating conditions. In other words, these adsorption sites were found to be completely hydrolyzed prior to the time of the first measurements.

For the second type of adsorption site, we return to Reaction 1 and write:

$$A_{02} = A_2 + EA_2 \quad \ldots\ldots\ldots\ldots (3)$$

where A_2 is the site not occupied by an enzyme. Also:

$$E_0 = E + EA_2 \quad \ldots\ldots\ldots\ldots (4)$$

The rate of enzyme complex formation and disappearance is given by:

$$\frac{d(EA_2)}{dt} = k_{ads}(E^* A_2) - k_{des}(EA_2) - k_3(EA_2) \quad \ldots\ldots (5)$$

and by substituting Equations 3 and 4 into Equation 5 and assuming pseudo-steady state:

$$k_{ads}(E_0 - EA_2)(A_{02} - EA_2) = k_{des}(EA_2) + k_3(EA_2) \quad \ldots\ldots (6)$$

If it is assumed that the enzyme is in large excess, $E_0 >> EA_2$, Equation 6 can be simplified to:

$$E_0 * A_{02} - E_0 * EA_2 = \left(\frac{k_{des} + k_3}{k_{ads}}\right) * EA_2 \quad \ldots\ldots\ldots (7)$$

Letting $K_A = (k_{des} + k_3)/k_{ads}$, Equation 7 then simplifies to the following expression for the complex concentration:

$$EA_2 = \frac{A_{02}E_0}{K_A + E_0} \quad \ldots\ldots\ldots\ldots (8)$$

The development is complete if it is assumed that hydrolysis is accomplished by irreversible decomposition of the EA_2 complex resulting in a decrease in the amount of slowly hydrolyzing starch granules, S_2:

$$\frac{dS_2}{dt} = -k_3 * EA_2 \quad \ldots\ldots\ldots\ldots (9)$$

Substituting Equation 8 into Equation 9:

$$\frac{dS_2}{dt} = \frac{-k_3 A_{02} E_0}{K_A + E_0} \quad \ldots\ldots\ldots\ldots (10)$$

The number of adsorption sites on the substrate is assumed proportional to the granule mass concentration ($A_{02} = K^* S_2$) giving:

$$\frac{dS_2}{dt} = \frac{-KS_2E_0}{K_A + E_0} \quad \ldots\ldots\ldots\ldots (11)$$

where $K = k_3K'$. This expression has the same form as that used by Tsuk and Oster (1961) and reported in textbooks (Bailey and Ollis, 1986). Integrating Equation 11 generates an equation for the substrate concentration at any time, t, and any enzyme concentration, E_0:

$$S_2 = \frac{S_{02}}{\exp\left(\frac{K * E_0 * t}{K_A + E_0}\right)} \quad \ldots\ldots\ldots\ldots (12)$$

The situation of $E_0 >> A_0$ is not at all uncommon for reactions involving solid substrates. In 1961, Tsuk and Oster completed an enzyme experiment on an insoluble substrate where E_0/A_0 was approximately 4000. In this work, enzyme concentrations greater than 1000 times those used in industry were explored. Over 10^{10} enzyme molecules were supplied per starch granule in the bioreactors, therefore it is believed that the assumption of E_0 being much greater than EA is valid in the above derivation.

Materials and methods

Materials

Three α-amylase enzymes were used in this study and were purchased from the Sigma chemical company. They were: (i) bacterial, catalogue #A-6814 from *Bacillus* species; (ii) fungal, catalogue #A-0273 from *Aspergillus oryzae*, and (iii) plant,

TABLE 1
Enzyme Activities and Costs

Type of Enzyme	Measured Activity (U/mg)**	Cost*** ($/kg)	Cost*** ($/U*10^3)
Accutrol	43	–	–
Bacterial	7.24	8600	1.19
Fungal	4.32	12000	2.78
Barley	0.058	420	7.24

**One unit of activity is the amount of enzyme that will produce 1 mole of product in 1 minute at pH 7 and 30°C. ***Based on 1995 Sigma Catalogue prices.

catalogue #A-2771 from barley. Wheat starch 4 was donated by CSP Mills, Saskatoon, but manufactured by Archer-Daniels-Midland/Ogilvie in Montreal. Wheat starch 4 is an A grade starch which contains mostly large granules above 18 microns in diameter according to the manufacturer. All additional chemicals used in this study were analytical grade or better.

HYDROLYSIS REACTIONS

The hydrolysis experiments were conducted in an environmental chamber maintained at constant temperatures using a temperature controller and heater. The reactions were carried out in well-mixed, 30 mL baffled batch reactors using aqueous solutions containing 0.02 M sodium acetate and 0.001 M calcium chloride. These solutions were adjusted to pH 4.5, 5.5 or 6.5 using hydrochloric acid. The enzyme was added to the reactor at concentrations from 0.5 to 30 mg/mL and the starch was added to start the reaction at concentrations from 3 to 100 mg/mL. Reaction samples were withdrawn at various time intervals, and the reaction was stopped by lowering the pH to 2.0 with 0.06 M HCl solution. This solution was then neutralized 30 minutes later with an equal amount of NaOH. This procedure was tested and found to immediately stop the hydrolysis reactions and did not interfere with any of the analytical measurements.

ANALYSIS

The relative activities of the three enzymes and a control standard were tested experimentally by hydrolyzing 4,6-ethylidene, (G_1)-p-nitrophenyl, (G_1)-α,d-maltoheptaside using an enzyme assay kit purchased from Sigma Chemicals (Catalogue #577). This hydrolysis reaction produces a solution with a red color which is detected spectrophotometrically at 405 nm.

The starch granule size distribution was measured using a Leeds and Northrup, Microtrac II (Model 7997-10) Particle Size Analyzer (PSA). This instrument uses the principle of light diffraction from a laser beam which is sent through a flowing aqueous slurry containing thousands of starch particles. Individual photographs of starch granules were also recorded as they were being hydrolyzed using a Phillips Model 505 scanning electron microscope.

A spectrophotometer was used to measure the insoluble starch concentration (LKB Novaspec II). The spectrophotometer absorbance at 600 nm was calibrated to the starch particle concentration using both fresh and partially hydrolyzed starch particles in reactor media. The data fell on a straight line regardless of the particle source (fresh or partially hydrolyzed particles) with a slope 3.2 ± 0.1 mg of particles/OD unit. The glucose concentration was measured using a YSI Model 23A Glucose Analyzer. The COD (chemical oxygen demand) was measured using a Hach model 45600 COD reactor. The measurement of dissolved carbon was conducted by oxidizing the sample in acidic agent vials (Hach catalogue #21259-15) at high temperatures. These vials permit COD measurements in the range 0 – 1500 ppm.

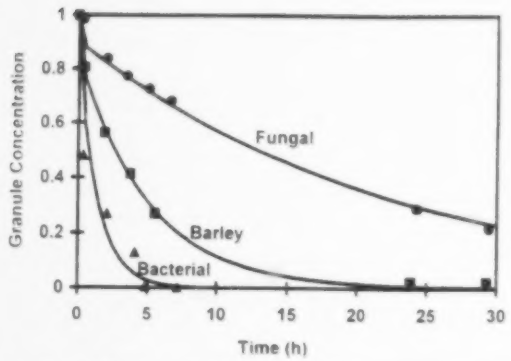

Figure 1 — Comparison of three amylase enzymes dissolving wheat starch granules (S_0 = 3 mg/mL, E_0 = 8 mg/mL, pH = 5.5, T = 26°C; concentrations are relative to initial concentrations; lines are Equation 13).

Results and discussion

Table 1 compares the cost of the three enzymes used in this study based on both the mass of the crude enzyme preparation and enzyme activity on soluble starch using the enzyme assay kit. It can be seen that the barley α-amylase has the lowest cost per kg but the bacterial enzyme was found to be 125 times more active and cost only 20 times more than barley α-amylase. Thus it appears that the most economical enzyme to hydrolyze soluble starch would be bacterial α-amylase which seems to be the most popular choice for industrial use.

Figure 1 compares the degradation of starch granules at 26°C, pH 5.5 and at 8 mg of crude enzyme preparation/mL for all three enzymes. The comparison is made at similar starting concentrations of starch granules and all data represent the average of 2 runs at that condition. In order to negate the effect of the slightly different initial starch concentrations, relative concentrations are shown by dividing each granule measurement by the measurement taken at time zero. At these conditions, it can be seen that the bacterial enzyme provided the fastest degradation of starch granules, achieving 95% solubilization in approximately 6 hours. In that same time period, the barley and fungal enzymes solubilized 75% and 30% of the starch granules, respectively. Sandstedt and Gates (1954) also found that the bacterial enzyme was the most rapid enzyme source for corn starch granules followed by fungal and barley enzymes.

Although the bacterial enzyme outperformed the barley enzyme under the conditions shown in Figure 1, the rate of granule hydrolysis by the bacterial enzyme was not 125 times faster as one might expect based on the enzyme activities reported in Table 1. Other researchers (Walker and Hope, 1963) have suggested the reason for enhanced activity of certain amylase enzymes is due to the affinity towards attaching to solid particle surfaces. Based on the very low cost of barley enzyme shown in Table 1 and the fact that it is within a factor of two times as active as the bacterial enzyme when applied to starch granules, it is clear that barley α-amylase will be the most economical source of enzyme to use in this process.

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